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Applicant LILIUS, Esa-Matti et al	

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(54) Title: RENILLA LUCIFERASE AND GREEN FLUORESCENT PROTEIN FUSION GENES

(57) Abstract

A fusion gene is provided comprising the cDNA of *Renilla* luciferase and the cDNA of the "humanized" *Aequorea* green fluorescent protein. The fusion gene was used to produce a novel protein, the "Renilla–GFP fusion protein", which displayed both the luciferase activity of *Renilla* luciferase, and the green fluorescence of GFP. The Renilla–GFP fusion gene is useful as a double marker for monitoring gene expression quantitatively in UV light and by enzyme activity.

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RENILLA LUCIFERASE AND GREEN FLUORESCENT PROTEIN FUSION GENES

CROSS-REFERENCE TO RELATED APPLICATIONS

The present Application is a International Application corresonding to United States Patent Application 08/771,850, filed December 23, 1996, entitled "The Construction and Expression of Renilla Luciferase and Green Fluorescent Protein Fusion Genes"; and is a Continuation-in-Part of United States Provisional Patent Application 60/027,657, filed October 4, 1996, entitled "The Construction and Expression of Renilla Luciferase and Green Fluorescent Fusion Genes in *E. coli* and Mammalian Cells," the contents of which are incorporated herein by reference in their entirety.

BACKGROUND

Green Fluorescent Protein (GFP) is a light emitting protein purified from the jellyfish *Aequorea victoria*. GFP can emit green light by accepting energy transfer from sources that include exogenous blue light and *Renilla* luciferase catalyzed reactions. The gene for GFP was cloned and its cDNA is a powerful reporter gene in a variety of living systems, including bacteria, fungi, and mammalian tissues. The UV light stimulated GFP fluorescence does not require cofactors and the gene product alone can be sufficient to allow detection of living cells under the light microscope.

By modifying the wild type GFP protein, red-shifted GFP variants with bright emission have also been produced. These variants include EGFP, GFPS65T and RSGF. Recently, GFP was expressed in a human cell line and *in vivo*. C. Kaether, H.H. Gerdes. Visualization of protein transport along the secretory pathway using green fluorescent protein. FEBS-Lett. 1995; 369:267-71. "Humanized" GFP was synthesized with nucleotide changes that did not change the amino acid sequences with one exception.

Renilla luciferase is an enzyme purified from *Renilla reniformis*. The enzyme catalyzes the oxidative decarboxylation of coelenterazine in the presence of oxygen to produce blue light with an emission wavelength maximum of 478 nm. In *Renilla reniformis* cells, however, this reaction is shifted toward the green with a wavelength maximum of 510 nm due to an energy transfer to a Green Fluorescent Protein.

The gene for *Renilla* luciferase (*ruc*) was cloned and its cDNA was shown to be useful as a reporter gene in various living systems. D.C. Prasher, V.K. Eckenrode, W.W. Ward, F.G. Prendergast, M.J. Cormier. Primary structure of the *Aequorea victoria* green-fluorescent protein. Gene 1992; 111:229-33. By providing appropriate promoters to the

cDNA as gene cassettes, the gene was expressed in bacteria, transformed plant cells, and mammalian cells. The high efficiency of *Renilla* luciferase is a useful trait as a marker enzyme for gene expression studies.

Given the properties of GFP and *Renilla* luciferase, it would be useful to have a single protein combining the functions of both *Renilla* luciferase enzymes and GFP to monitor gene expression quantitatively by UV light excitation or qualitatively by enzyme activity measurements.

SUMMARY

According to one embodiment of the present invention, there are provided fusion gene constructs comprising the cDNA of *Renilla* luciferase and the cDNA of the "humanized" *Aequorea* green fluorescent protein. The fusion gene constructs were used to transform both prokaryotic and eukaryotic cells. One construct was expressed as a polypeptide having a molecular weight of about 65 kDa. This polypeptide, the "Renilla-GFP fusion protein," was bifunctional, displaying both the luciferase activity of *Renilla* luciferase and the green fluorescence of GFP. The Renilla-GFP fusion gene is useful as a double marker for monitoring gene expression in living cells and quantitatively by enzymatic activity.

The invention includes a protein comprising a polypeptide having both luciferase and GFP activities, or biologically active variants of a polypeptide having both luciferase and GFP, or a protein recognized by a monoclonal antibody having affinity to the polypeptide having both luciferase and GFP activities. The polypeptide can be made by recombinant DNA methods.

The invention further includes a high affinity monoclonal antibody that immunoreacts with the polypeptide. The antibody can have an Fc portion selected from the group consisting of the IgM class, the IgG class and the IgA class. The invention also includes a high affinity monoclonal antibody that immunoreacts with a polypeptide having both luciferase and GFP activities.

The invention further includes a polynucleotide sequence coding for a polypeptide having both luciferase and GFP activities, or its complementary strands, and a polynucleotide sequence that hybridizes to such a sequence and that codes on expression for a polypeptide having both luciferase and GFP activities, or its complementary strands.

The invention further includes a purified and isolated DNA molecule comprising a polynucleotide coding for a polypeptide having both luciferase and GFP

activities, or its complementary strands. The polynucleotide can comprise the sequence as set forth in SEQ ID NO:1.

The invention further includes a vector containing a DNA molecule coding for a polypeptide having both luciferase and GFP activities. The polynucleotide can comprise the sequence as set forth in SEQ ID NO:1. The vector can be used to stably transform or transiently transfect a host cell.

The invention further includes a method of making a polypeptide having both luciferase and GFP activities. The method comprises the steps of, first, culturing a microorganism transformed with a polynucleotide vector containing a gene cassette coding for a polypeptide having both luciferase and GFP activities. Next, the polypeptide having both luciferase and GFP activities is recovered.

The invention further includes a method of quantifying promoter activations and GFP fluorescence based on luciferase activity measurements. The method comprises the step of providing the polypeptide according to the present invention.

The invention further includes a method of making a monoclonal antibody that immunoreacts with a polypeptide having both luciferase and GFP activities. The method comprises the steps of, first, administering to a host a polypeptide having both luciferase and GFP activities in an amount sufficient to induce the production of antibodies to the polypeptide from the host's antibody-producing cells. Next, the antibody-producing cells are recovered from the host. Then, cell hybrids are formed by fusing the antibody-producing cell to cells capable of substantially unlimited reproduction. Then, the hybrids are cultured. Next, the monoclonal antibodies are collected as a product of the hybrids.

The invention further includes a method of monitoring gene expression quantitatively and qualitatively in a cell using a gene fusion construct coding for a polypeptide having both luciferase and GFP activities. The method comprises the steps of, first, providing a gene fusion construct coding for a polypeptide having both Renilla luciferase and GFP activity. Next, the gene fusion construct is introduced into the cell. Then, the cell containing the gene fusion construct is maintained in a manner allowing the cell to express the polypeptide. Then, the cell is measured for luciferase and fluorescent activity. The construct can include a polynucleotide sequence as set forth in SEQ ID NO:1.

The invention further includes a method of monitoring gene expression quantitatively and qualitatively in a cell using a gene fusion construct coding for a polypeptide

having both luciferase and GFP activities. The method comprises the steps of, first, providing a gene fusion construct coding for a polypeptide having both luciferase and GFP activities. Next, the gene fusion construct is introduced into the cell. Then, the cell containing the gene fusion construct is maintained in a manner allowing the cell to express the polypeptide. Next, the cell is measured for luciferase and fluorescent activity.

FIGURES

These and other features, aspects and advantages of the present invention will become better understood with regard to the following description, appended claims, and accompanying figures where:

Figure 1 is a schematic diagram showing the construction of a *Renilla* luciferase and "humanized" GFP fusion gene cassette according to the present invention for gene expression in *E. coli* where "RG," top, is the fusion gene cassette with the *Renilla* luciferase coding sequence (ruc) at the 5' terminus, and "GR," bottom, is the fusion gene cassette with the GFP coding sequence (gfp_h) at the 5' terminus;

Figure 2 is a schematic diagram showing the construction of *Renilla* luciferase and "humanized" GFP fusion gene cassette according to the present invention for gene expression in mammalian cells where "RG," top, is the fusion gene cassette with the *Renilla* luciferase coding sequence (ruc) at the 5' terminus, and "GR," bottom, is the fusion gene cassette with the GFP coding sequence (gfp_h) at the 5' terminus;

Figure 3 is a map of the plasmids used for cloning and expression of the RG gene construct in *E. coli* (top) and the GR gene construct in *E. coli* (bottom);

Figure 4 is a map of the plasmids used for cloning and expression of the RG gene construct in mammalian systems (top) and the GR gene construct in mammalian systems (bottom);

Figure 5 are photomicrographs of cells transformed by the fusion genes using fluorescence microscopy and fluorescence imaging to show GFP activity;

Figure 6 are bar graphs of luciferase activity of the fusion gene constructs in *E. coli* (top) and mammalian cells (bottom);

Figure 7 is a spectroscopic measurement of *Renilla* luciferase activity and GFP activity in *E. coli*;

Figure 8 is a Western blot showing the detection of fusion gene expression in *E. coli* using anti-*Renilla* luciferase antibody;

Figure 9 are photomicrographs of mouse embryonic stem cells using fluorescence image analysis demonstrating the expression of the RG fusion gene; and

Figure 10 are photomicrographs of mouse embryos using fluorescence image analysis demonstrating the expression of the RG fusion gene.

DESCRIPTION

According to one embodiment of the present invention, there is provided a fusion gene comprising the cDNA of *Renilla* luciferase and the cDNA of the "humanized" *Aequorea* green fluorescent protein. According to another embodiment of the present invention, there is provided a single polypeptide that exhibits both *Renilla* luciferase and GFP activities. This bifunctional polypeptide can facilitate the identification of transformed cells at the single cell level, in cell cultures, transformed tissues and organs based on fluorescence of the polypeptide. At the same time, the polypeptide can also be used to quantify promoter activations and GFP fluorescence based on luciferase activity measurements.

The cDNA of *Renilla reniformis* luciferase (ruc) has been cloned and used successfully as a marker gene in a variety of transgenic species. See, for example, Lorenz, W.W. McCann, R.O., Longiaru, M. and Cormier, M.J. Isolation and expression of a cDNA encoding *Renilla reniformis* luciferase. Proc. Natl. Acad. Sci. USA 1991; 88:4438-4442; Mayerhofer, R., Langridge, W.H.R., Cormier, M.J., and Szalay, A.A. Expression of recombinant *Renilla* luciferase in transgenic plants results in high levels of light emission. The Plant Journal 1995; 7:1031-1038; and Lorenz, W.W., Cormier, M.J., O'Kane, D.J., Hua, D., Escher, A. A.Szalay, A.A. Expression of the *Renilla reniformis* luciferase gene in mammalian cells. J. Biolumin. Chemilumin. 1995; 11:31-37, incorporated herein by reference in their entirety. Similarly, the transfer and expression of Green-Fluorescent-Protein (GFP) cDNA from *Aequorea victoria* resulted in high levels of GFP in transformed cells that allowed convenient visualization of individual cells under the microscope. See, for example, Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W. and Prasher, D.C. Green fluorescent protein as a marker for gene expression. Science 1994; 263:802-805, incorporated herein by reference in its entirety.

The present invention involves the production of fusion genes from the cDNA of *Renilla* (ruc) and the cDNA of the "humanized" *Aequorea* GFP (gfp_h). A description of "humanized" *Aequorea* GFP (gfp_h) can be found, for example, in Zolotukhin, S., Potter, M., and Huaswirth, W.W., Guy, J., and Muzyczka, N. A "humanized" green fluorescent protein

cDNA adapted for high-level expression in mammalian cells. J. Virology 1996; 70:4646-4654, incorporated herein by reference in its entirety.

The first fusion gene, designated the "RG fusion gene," SEQ ID NO:1 and shown at the top of Figures 1 and 2, contains the *Renilla* cDNA linked at the modified 3' end to a fifteen polynucleotide linker sequence encoding five amino acids, Ala-Ala-Ala-Ala-Thr, residues 312-316 of SEQ ID NO:1, followed by the 5' end of the intact GFP cDNA. The second fusion gene, designated the "GR fusion gene," SEQ ID NO:2 and shown at the bottom of Figures 1 and 2, contains the cDNA of GFP linked to a twenty-seven polynucleotide linker sequence encoding nine amino acids, Gly-Try-Gln-Ile-Glu-Phe-Ser-Leu-Lys, residues 239-247 of SEQ ID NO:2, followed by the 5' end of *Renilla* cDNA. Both genes were placed into prokaryotic pGEM-5zf(+) and eukaryotic pCEP4 expression vectors, and transformed into *E. coli*, and various mammalian cell lines, and microinjected into mouse embryos. PT_7 was the bacterial T7 promoter used for gene expression. P_{cmv} was the CMV promoter used for gene expression in mouse fibroblast cells, embryonic stem cells and mouse embryos.

Unexpectedly, only cells transformed with the RG fusion gene gave strong fluorescence while the cells containing the GR fusion gene exhibited minimal response to UV light under the microscope. In contrast, luciferase measurements in homogenates of cells transformed with RG gene cassettes or with GR gene cassettes were indistinguishable from each other in both bacterial and mammalian cells. Further, spectrofluorimeter data indicated that there was energy transfer between *Renilla* luciferase and GFP in the RG fusion gene containing cells but did not indicate such energy transfer in cells containing the GR fusion gene. The protein expressed in the RG fusion gene containing cells was analyzed and found to be a 65 kDa polypeptide. A detailed description of the construction and expression of the fusion genes, and analyses of their protein products is given below.

Production of the Fusion Gene Constructs:

The vectors used for cloning and expression of the gene constructs in *E. coli* and mammalian systems were pGEM-5zf(+) (Promega) and pCEP4, respectively. Figure 3 is a map of the plasmids used for cloning and expression of the RG gene construct in *E. coli*, pGEM-5zf(+)-RG (top) and the map of the plasmids used for cloning and expression of the GR gene construct in *E. coli*, pGEM-5zf(+)-GR (bottom). Both were under the transcriptional control of T7 promoter. The *E. coli* strains which were transformed were DLT101 and DH5α.

Similarly, Figure 4 is a map of the plasmids used for cloning and expression of the RG gene construct in mammalian systems, pCEP4-RG (top), and a map of the plasmids used for cloning and expression of the GR gene construct in mammalian systems, pCEP4-GR (bottom). Both were under the transcriptional control of CMV promoter. The mammalian cell line that was transformed was LM-TK$^-$ embryonic stem cells and embryos.

Five primers were designed for cloning the RG and GR gene constructs. Single underlines indicate Shine-Dalgarno sequences. Double underlines indicate the restriction sites. The start codons are in bold. Sequences in bold italics indicate the removal of stop codons from both *ruc* and gfp_h genes.

Primer 1, SEQ ID NO:3: RUC5: 5'CTGCAG (PstI)
AGGAGGAATTCAGCTTAAAGATG3'

Primer 2, SEQ ID NO:4: RUC3: 5'GCGGCCGC (Not I) ***TTG*** TTCATTTTTGAGAAC3'

Primer 3, SEQ ID NO:5: GFP5:5'GGGGTACC (KpnI)
CCATGAGCAAGGGCGAGGAACT3'

Primer 4, SEQ ID NO:6: GFP3: 5'GGGGTACC (KpnI)
C***CTT***GTACAGCTCGTCCATGCCA3'

Primer 5, SEQ ID NO:7: GFP5a 5' CCCGGG (SmaI)
AGGAGGTACCCCATGAGCAAG3'.

The *Renilla* luciferase-GFP fusion gene (RG gene cassette) and the GFP-*Renilla* luciferase fusion gene (GR gene cassette) were constructed by removing the stop codons, and by adding restriction sites and Shine-Dalgarno sequences to the 5' end of the cDNAs using PCR according to techniques known to those with skill in the art. The PCR products were cloned using the pGEM-T system (Promega Corporation, Madison, WI). Primers were designed so that the downstream cDNA is in frame with the upstream cDNA. The linker sequences are shown in Figures 1 and 2 and described above. After cloning, the RG and GR gene cassettes were under the transcriptional control of T7 in pGEM-5zf(+) vector and CMV in pCEP4 vector, which were used for expression in *E. coli* and mammalian cells, respectively.

Determination of activity of fusion genes and their corresponding protein products:

GFP activity *in vivo* was visualized as follows. *E. coli* strain DH5α was transformed with the plasmids pGEM-5zf(+)-RG and pGEM-5zf(+)-GR. Positive colonies were identified and cultured in LB medium with 100 μg/ml of ampicillin selection, according

to techniques known to those with skill in the art. Twelve hours later, one drop of *E. coli* culture was put on a slide and visualized by fluorescent microscopy at 1000 x magnification. LM-TK$^-$ cells were transfected with plasmids pCEP4-RG and pCEP4-GR using calcium phosphate methods known to those with skill in the art. The culture dishes were monitored using an inverted fluorescent microscope 12 hours after the transfection.

Luciferase activity was assayed as follows. An aliquot of transformed *E. coli* was used for a luciferase assay in a Turner TD 20e luminometer (Turner Designs, Sunnyvale, CA), both before and after IPTG induction. The results were recorded as relative light units. Mammalian cells harvested 36 hrs after transfection were measured for luciferase activity.

Corrected emission spectra were detected spectrofluorimetrically using a SPEX fluorolog spectrofluorimeter operated in the ratio mode. Fluorescence emission was excited at 390 nm. Bioluminescence emission was recorded with the excitation beam blocked following the addition of 0.1 μg of coelenterazine in acidified methanol. Five spectra were averaged for each sample over a wavelength range from 400 to 600 nm.

The fusion proteins were isolated and immunoactivity detected as follows. 1 ml of *E. coli* (OD_{600}=1.0) was harvested. 400 μl of cell suspension buffer (0.1M NaCl, 0.01 M Tris-HCl pH 7.6, 0.001 M EDTA, 100 μg/ml PMSF) and 100 μl of loading buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol) were added. The samples were boiled for 4 min and loaded to a 7.5%-20% gradient SDS-polyacrylamide gel. Polyclonal anti-*Renilla* luciferase was used as the primary antibody for detection and goat peroxidase anti-IgG (anti-rabbit) as the secondary antibody.

Referring now to Figure 5, there are shown photomicrographs of GFP activity in transformed *E. coli* cells (5A, left side) and LM-TK$^-$ mouse fibroblast cells (5B, right side) by fluorescence microscopy and fluorescence imaging. As can be seen, individual *E. coli* cells and mammalian cells transformed with the RG fusion gene construct exhibited strong green fluorescence under oil immersion.

Referring now to Figure 6, there are shown bar graphs of luciferase activity of the gene constructs in *E. coli* (top) and mammalian cells (bottom). The white bars indicate activity before promoter induction. The black bars indicate activity after promoter induction. As can be seen, cells transformed with the RG fusion gene construct have significant luciferase activity, which is reduced 3-fold in the cells transformed with the GR fusion gene construct.

Referring now to Figure 7, there is shown a spectroscopic measurement of *Renilla* luciferase activity and GFP activity in *E. coli* transformed with various gene constructs. As can be seen, cells containing *Renilla* luciferase gene (short dashes) show only one emission peak at approximately 478 nm. Cells containing the GR gene fusion construct (light solid) also show one emission peak at approximately 478 nm, indicating *Renilla* luciferase activity only. By contrast, cells containing the RG gene fusion construct (heavy solid) show an emission peak at approximately 510 nm with excitation at 390 nm. Cells containing the RG gene fusion construct with the addition of coelanterizine (long dashes) show emission peaks at both approximately 478 nm and 510 nm indicating that the energy transfer between *Renilla* luciferase and GFP occurred in these cells. The lack of GFP activity in GR gene cassette transformed cell lines could be due to incorrect folding, due to the requirement for a free GFP C-terminus, or due to interference of the linker polypeptide with GFP activity in the fusion protein, among other possible explanations.

Referring now to Figure 8, there is shown a western blot used to detect fusion gene expression in *E. coli* using anti-*Renilla* luciferase antibody. Reading from left to right, the "C" lane shows the total protein extracted from non-transformed *E. coli* cells. The "R" lane shows the total protein extracted from *E. coli* cells transformed with the ruc gene alone. The "G" lane shows the total protein extracted from *E. coli* cells transformed with the gfp_h gene alone. The "RG" lane shows the total protein extracted from *E. coli* cells transformed with the RG fusion gene cassette. The "GR" lane shows the total protein extracted from *E. coli* cells transformed with the GR fusion gene cassette.

As can be seen, protein extracted from *E. coli* cells transformed with the ruc gene alone produced a band with a molecular weight of about 34 kDa. Protein extracted from *E. coli* cells transformed with the RG fusion gene cassette produced a band with a molecular weight of about 65 kDa. Protein extracted from *E. coli* cells transformed with the GR fusion gene cassette produced a band with a molecular weight of about 34 kDa. These data imply that cells transformed with the GR fusion gene cassette produced luciferase but did not produce fusion protein. Such a lack of fusion protein production by cells transformed with the GR fusion cassette would explain the lack of green fluorescent activity in these cells.

Referring now to Figure 9, there are shown photomicrographs using fluorescence image analysis demonstrating the expression of the RG fusion gene in mouse

embryonic stem cells transformed by electroporation procedures. Transformed colonies were selected based on GFP activity under fluorescence microscopy.

Referring now to Figure 10, there are shown photomicrographs using fluorescence image analysis demonstrating the expression of the RG fusion genes in mouse embryos. The embryos were injected with the linearized RG plasmid, and *in vitro* cultured. The expression of GFP activity was monitored daily by fluorescent microscope and recorded by an imaging collection system.

Based on this data, we conclude that the RG fusion construct disclosed herein can be expressed in both prokaryotic and eukaryotic cells to produce a bifunctional polypeptide that exhibits both *Renilla* luciferase and GFP activity. This bifunctional polypeptide is a useful tool for identification of transformed cells at the single cell level based on fluorescence. It allows the simultaneous quantification of promoter activation in transformed tissues and transgenic organisms by measuring luciferase activity. The dual function of this protein allows the monitoring of bacterial cells in their living hosts and the differentiation of cells in the developing embryo and throughout the entire animal.

Although the present invention has been discussed in considerable detail with reference to certain preferred embodiments, other embodiments are possible. Therefore, the spirit and scope of the appended claims should not be limited to the description of preferred embodiments contained herein.

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(iii) NUMBER OF SEQUENCES: 7
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(v) COMPUTER READABLE FORM:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
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             20                  25                  30

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Phe Ile Asn Tyr Tyr Asp Ser Glu Lys His Ala Glu Asn Ala Val Ile
         35                  40                  45

TTT TTA CAT GGT AAC GCG GCC TCT TCT TAT TTA TGG CGA CAT GTT GTG       192
Phe Leu His Gly Asn Ala Ala Ser Ser Tyr Leu Trp Arg His Val Val
     50                  55                  60

CCA CAT ATT GAG CCA GTA GCG CGG TGT ATT ATA CCA GAT CTT ATT GGT       240
Pro His Ile Glu Pro Val Ala Arg Cys Ile Ile Pro Asp Leu Ile Gly
 65                  70                  75                  80

ATG GGC AAA TCA GGC AAA TCT GGT AAT GGT TCT TAT AGG TTA CTT GAT       288
Met Gly Lys Ser Gly Lys Ser Gly Asn Gly Ser Tyr Arg Leu Leu Asp
                 85                  90                  95
```

```
CAT TAC AAA TAT CTT ACT GCA TGG TTT GAA CTT CTT AAT TTA CCA AAG        336
His Tyr Lys Tyr Leu Thr Ala Trp Phe Glu Leu Leu Asn Leu Pro Lys
            100                 105                 110

AAG ATC AAT TTT GTC GGC CAT GAT TGG GGT GCT TGT TTG GCA TTT CAT        384
Lys Ile Ile Phe Val Gly His Asp Trp Gly Ala Cys Leu Ala Phe His
        115                 120                 125

TAT AGC TAT GAG CAT CAA GAT AAG ATC AAA GCA ATA GTT CAC GCT GAA        432
Tyr Ser Tyr Glu His Gln Asp Lys Ile Lys Ala Ile Val His Ala Glu
    130                 135                 140

AGT GTA GTA GAT GTG ATT GAA TCA TGG GAT GAA TGG CCT GAT ATT GAA        480
Ser Val Val Asp Val Ile Glu Ser Trp Asp Glu Trp Pro Asp Ile Glu
145                 150                 155                 160

GAA GAT ATT GCG TTG ATC AAA TCT GAA GAA GGA GAA AAA ATG GTT TTG        528
Glu Asp Ile Ala Leu Ile Lys Ser Glu Glu Gly Glu Lys Met Val Leu
                165                 170                 175

GAG AAT AAC TTC TTC GTG GAA ACC ATG TTG CCA TCA AAA ATC ATG AGA        576
Glu Asn Asn Phe Phe Val Glu Thr Met Leu Pro Ser Lys Ile Met Arg
            180                 185                 190

AAG TTA GAA CCA GAA GAA TTT GCA GCA TAT CTT GAA CCA TTC AAA GAG        624
Lys Leu Glu Pro Glu Glu Phe Ala Ala Tyr Leu Glu Pro Phe Lys Glu
        195                 200                 205

AAA GGT GAA GTT CGT CGT CCA ACA TTA TCA TGG CCT CGT GAA ATC CCG        672
Lys Gly Glu Val Arg Arg Pro Thr Leu Ser Trp Pro Arg Glu Ile Pro
    210                 215                 220

TTA GTA AAA GGT GGT AAA CCT GAC GTT GTA CAA ATT GTT AGG AAT TAT        720
Leu Val Lys Gly Gly Lys Pro Asp Val Val Gln Ile Val Arg Asn Tyr
225                 230                 235                 240

AAT GCT TAT CTA CGT GCA AGT GAT GAT TTA CCA AAA ATG TTT ATT GAA        768
Asn Ala Tyr Leu Arg Ala Ser Asp Asp Leu Pro Lys Met Phe Ile Glu
                245                 250                 255

TCG GAT CCA GGA TTC TTT TCC AAT GCT ATT GTT GAA GGC GCC AAG AAG        816
Ser Asp Pro Gly Phe Phe Ser Asn Ala Ile Val Glu Gly Ala Lys Lys
            260                 265                 270

TTT CCT AAT ACT GAA TTT GTC AAA GTA AAA GGT CTT CAT TTT TCG CAA        864
Phe Pro Asn Thr Glu Phe Val Lys Val Lys Gly Leu His Phe Ser Gln
        275                 280                 285

GAA GAT GCA CCT GAT GAA ATG GGA AAA TAT ATC AAA TCG TTC GTT GAG        912
Glu Asp Ala Pro Asp Glu Met Gly Lys Tyr Ile Lys Ser Phe Val Glu
    290                 295                 300

CGA GTT CTC AAA AAT GAA CAA GCG GCC GCC GCC ACC ATG AGC AAG GGC        960
Arg Val Leu Lys Asn Glu Gln Ala Ala Ala Ala Thr Met Ser Lys Gly
305                 310                 315                 320

GAG GAA CTG TTC ACT GGC GTG GTC CCA ATT CTC GTG GAA CTG GAT GGC       1008
Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val Glu Leu Asp Gly
                325                 330                 335

GAT GTG AAT GGG CAC AAA TTT TCT GTC AGC GGA GAG GGT GAA GGT GAT       1056
Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu Gly Glu Gly Asp
            340                 345                 350
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GCC ACA TAC GGA AAG CTC ACC CTG AAA TTC ATC TGC ACC ACT GGA AAG      1104
Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys Thr Thr Gly Lys
        355                 360                 365

CTC CCT GTG CCA TGG CCA ACA CTG GTC ACT ACC TTC ACC TAT GGC GTG      1152
Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Phe Thr Tyr Gly Val
    370                 375                 380

CAG TGC TTT TCC AGA TAC CCA GAC CAT ATG AAG CAG CAT GAC TTT TTC      1200
Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Gln His Asp Phe Phe
385                 390                 395                 400

AAG AGC GCC ATG CCC GAG GGC TAT GTG CAG GAG AGA ACC ATC TTT TTC      1248
Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe
                405                 410                 415

AAA GAT GAC GGG AAC TAC AAG ACC CGC GCT GAA GTC AAG TTC GAA GGT      1296
Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu Gly
            420                 425                 430

GAC ACC CTG GTG AAT AGA ATC GAG CTG AAG GGC ATT GAC TTT AAG GAG      1344
Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu
        435                 440                 445

GAT GGA AAC ATT CTC GGC CAC AAG CTG GAA TAC AAC TAT AAC TCC CAC      1392
Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn Tyr Asn Ser His
    450                 455                 460

AAT GTG TAC ATC ATG GCC GAC AAG CAA AAG AAT GGC ATC AAG GTC AAC      1440
Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly Ile Lys Val Asn
465                 470                 475                 480

TTC AAG ATC AGA CAC AAC ATT GAG GAT GGA TCC GTG CAG CTG GCC GAC      1488
Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val Gln Leu Ala Asp
                485                 490                 495

CAT TAT CAA CAG AAC ACT CCA ATC GGC GAC GGC CCT GTG CTC CTC CCA      1536
His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro Val Leu Leu Pro
            500                 505                 510

GAC AAC CAT TAC CTG TCC ACC CAG TCT GCC CTG TCT AAA GAT CCC ACC      1584
Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser Lys Asp Pro Asn
        515                 520                 525

GAA AAG AGA GAC CAC ATG GTC CTG CTG GAG TTT GTG ACC GCT GCT GGG      1632
Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val Thr Ala Ala Gly
    530                 535                 540

ATC ACA CAT GGC ATG GAC GAG CTG TAC AAG TGA                          1665
Ile Thr His Gly Met Asp Glu Leu Tyr Lys
545                 550
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1677 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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ATG AGC AAG GGC GAG GAA CTG TTC ACT GGC GTG GTC CCA ATT CTC GTG       48
Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val
  1               5                  10                  15
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GAA CTG GAT GGC GAT GTG AAT GGG CAC AAA TTT TCT GTC AGC GGA GAG          96
Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu
            20                  25                  30

GGT GAA GGT GAT GCC ACA TAC GGA AAG CTC ACC CTG AAA TTC ATC TGC         144
Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys
        35                  40                  45

ACC ACT GGA AAG CTC CCT GTG CCA TGG CCA ACA CTG GTC ACT ACC TTC         192
Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Phe
    50                  55                  60

ACC TAT GGC GTG CAG TGC TTT TCC AGA TAC CCA GAC CAT ATG AAG CAG         240
Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Gln
65                  70                  75                  80

CAT GAC TTT TTC AAG AGC GCC ATG CCC GAG GGC TAT GTG CAG GAG AGA         288
His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg
                85                  90                  95

ACC ATC TTT TTC AAA GAT GAC GGG AAC TAC AAG ACC CGC GCT GAA GTC         336
Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val
            100                 105                 110

AAG TTC GAA GGT GAC ACC CTG GTG AAT AGA ATC GAG CTG AAG GGC ATT         384
Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile
        115                 120                 125

GAC TTT AAG GAG GAT GGA AAC ATT CTC GGC CAC AAG CTG GAA TAC AAC         432
Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn
    130                 135                 140

TAT AAC TCC CAC AAT GTG TAC ATC ATG GCC GAC AAG CAA AAG AAT GGC         480
Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly
145                 150                 155                 160

ATC AAG GTC AAC TTC AAG ATC AGA CAC AAC ATT GAG GAT GGA TCC GTG         528
Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val
                165                 170                 175

CAG CTG GCC GAC CAT TAT CAA CAG AAC ACT CCA ATC GGC GAC GGC CCT         576
Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro
            180                 185                 190

GTG CTC CTC CCA GAC AAC CAT TAC CTG TCC ACC CAG TCT GCC CTG TCT         624
Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser
        195                 200                 205

AAA GAT CCC AAC GAA AAG AGA GAC CAC ATG GTC CTG CTG GAG TTT GTG         672
Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val
    210                 215                 220

ACC GCT GCT GGG ATC ACA CAT GGC ATG GAC GAG CTG TAC AAG GGG TAC         720
Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys Gly Tyr
225                 230                 235                 240

CAG ATC GAA TTC AGC TTA AAG ATG ACT TCG AAA GTT TAT GAT CCA GAA         768
Gln Ile Glu Phe Ser Leu Lys Met Thr Ser Lys Val Tyr Asp Pro Glu
                245                 250                 255

CAA AGG AAA CGG ATG ATA ACT GGT CCG CAG TGG TGG GCC AGA TGT AAA         816
Gln Arg Lys Arg Met Ile Thr Gly Pro Gln Trp Trp Ala Arg Cys Lys
            260                 265                 270

CAA ATG AAT GTT CTT GAT TCA TTT ATT AAT TAT TAT GAT TCA GAA AAA         864
Gln Met Asn Val Leu Asp Ser Phe Ile Asn Tyr Tyr Asp Ser Glu Lys
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        275                 280                 285

CAT GCA GAA AAT GCT GTT ATT TTT TTA CAT GGT AAC GCG GCC TCT TCT      912
His Ala Glu Asn Ala Val Ile Phe Leu His Gly Asn Ala Ala Ser Ser
    290                 295                 300

TAT TTA TGG CGA CAT GTT GTG CCA CAT ATT GAG CCA GTA GCG CGG TGT      960
Tyr Leu Trp Arg His Val Val Pro His Ile Glu Pro Val Ala Arg Cys
305                 310                 315                 320

ATT ATA CCA GAT CTT ATT GGT ATG GGC AAA TCA GGC AAA TCT GGT AAT     1008
Ile Ile Pro Asp Leu Ile Gly Met Gly Lys Ser Gly Lys Ser Gly Asn
                325                 330                 335

GGT TCT TAT AGG TTA CTT GAT CAT TAC AAA TAT CTT ACT GCA TGG TTT     1056
Gly Ser Tyr Arg Leu Leu Asp His Tyr Lys Tyr Leu Thr Ala Trp Phe
            340                 345                 350

GAA CTT CTT AAT TTA CCA AAG AAG ATC ATT TTT GTC GGC CAT GAT TGG     1104
Glu Leu Leu Asn Leu Pro Lys Lys Ile Ile Phe Val Gly His Asp Trp
        355                 360                 365

GGT GCT TGT TTG GCA TTT CAT TAT AGC TAT GAG CAT CAA GAT AAG ATC     1152
Gly Ala Cys Leu Ala Phe His Tyr Ser Tyr Glu His Gln Asp Lys Ile
    370                 375                 380

AAA GCA ATA GTT CAC GCT GAA AGT GTA GTA GAT GTG ATT GAA TCA TGG     1200
Lys Ala Ile Val His Ala Glu Ser Val Val Asp Val Ile Glu Ser Trp
385                 390                 395                 400

GAT GAA TGG CCT GAT ATT GAA GAA GAT ATT GCG TTG ATC AAA TCT GAA     1248
Asp Glu Trp Pro Asp Ile Glu Glu Asp Ile Ala Leu Ile Lys Ser Glu
                405                 410                 415

GAA GGA GAA AAA ATG GTT TTG GAG AAT AAC TTC TTC GTG GAA ACC ATG     1296
Glu Gly Glu Lys Met Val Leu Glu Asn Asn Phe Phe Val Glu Thr Met
            420                 425                 430

TTG CCA TCA AAA ATC ATG AGA AAG TTA GAA CCA GAA GAA TTT GCA GCA     1344
Leu Pro Ser Lys Ile Met Arg Lys Leu Glu Pro Glu Glu Phe Ala Ala
        435                 440                 445

TAT CTT GAA CCA TTC AAA GAG AAA GGT GAA GTT CGT CGT CCA ACA TTA     1392
Tyr Leu Glu Pro Phe Lys Glu Lys Gly Glu Val Arg Arg Pro Thr Leu
    450                 455                 460

TCA TGG CCT CGT GAA ATC CCG TTA GTA AAA GGT GGT AAA CCT GAC GTT     1440
Ser Trp Pro Arg Glu Ile Pro Leu Val Lys Gly Gly Lys Pro Asp Val
465                 470                 475                 480

GTA CAA ATT GTT AGG AAT TAT AAT GCT TAT CTA CGT GCA AGT GAT GAT     1488
Val Gln Ile Val Arg Asn Tyr Asn Ala Tyr Leu Arg Ala Ser Asp Asp
                485                 490                 495

TTA CCA AAA ATG TTT ATT GAA TCG GAT CCA GGA TTG TTT TCC AAT GCT     1536
Leu Pro Lys Met Phe Ile Glu Ser Asp Pro Gly Phe Phe Ser Asn Ala
            500                 505                 510

ATT GTT GAA GGC GCC AAG AAG TTT CCT AAT ACT GAA TTT GTC AAA GTA     1584
Ile Val Glu Gly Ala Lys Lys Phe Pro Asn Thr Glu Phe Val Lys Val
        515                 520                 525

AAA GGT CTT CAT TTT TCG CAA GAA GAT GCA CCT GAT GAA ATG GGA AAA     1632
Lys Gly Leu His Phe Ser Gln Glu Asp Ala Pro Asp Glu Met Gly Lys
    530                 535                 540
```

TAT ATC AAA TCG TTC GTT GAG CGA GTT CTC AAA AAT GAA CAA TAA 1677
Tyr Ile Lys Ser Phe Val Glu Arg Val Leu Lys Asn Glu Gln ***
545 550 555

(3) INFORMATION FOR SEQ ID NO:3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTGCAGAGGA GGAATTCAGC TTAAAGATG 29

(4) INFORMATION FOR SEQ ID NO:4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION:SEQ ID NO:4:

GCGGCCGCTT GTTCATTTTT GAGAAC 26

(5) INFORMATION FOR SEQ ID NO:5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION:SEQ ID NO:5:

GGGGTACCCC ATGAGCAAGG GCGAGGAACT 30

(6) INFORMATION FOR SEQ ID NO:6:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION:SEQ ID NO:6:

GGGGTACCCC TTGTACAGCT CGTCCATGCC A 31

(7) INFORMATION FOR SEQ ID NO:7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION:SEQ ID NO:7:

CCCGGGAGGA GGTACCCCAT GAGCAAG 27

WE CLAIM:

1. A protein comprising a polypeptide having both luciferase and GFP activities or biologically active variants thereof.

2. A recombinant protein according to claim 1.

3. A protein according to claim 1, having an amino acid sequence as set forth in SEQ ID NO:1.

4. A high affinity monoclonal antibody which immunoreacts with the polypeptide of claim 1.

5. The antibody of claim 4 having an Fc portion selected from the group consisting of the IgM class, the IgG class and the IgA class.

6. A protein recognized by a monoclonal antibody having affinity to the polypeptide of claim 1.

7. The protein of claim 1 in purified and isolated form.

8. A DNA sequence coding for a protein according to claim 1, or its complementary strands.

9. A DNA sequence which hybridizes to a DNA sequence according to claim 8 and which codes on expression for a polypeptide having both luciferase and GFP activities, or its complementary strands.

10. A high affinity monoclonal antibody which immunoreacts with a polypeptide having both luciferase and GFP activities.

11. A purified and isolated DNA molecule comprising a polynucleotide coding for a polypeptide having both luciferase and GFP activities, or its complementary strands.

12. The DNA of claim 11, wherein the polynucleotide comprises the sequence as set forth in SEQ ID NO:1.

13. A vector containing a DNA molecule coding for a polypeptide having both luciferase and GFP activities.

14. The vector of claim 13, wherein the polynucleotide comprises the sequence as set forth in SEQ ID NO:1.

15. A prokaryotic or eukaryotic host cell stably transformed or transfected by the vector of claim 13.

16. A method of making a polypeptide having both luciferase and GFP activities, the method comprising the steps of:

(a) culturing a microorganism transformed with a polynucleotide coding for a polypeptide having both luciferase and GFP activities; and

(b) recovering the polypeptide having both luciferase and GFP activities.

17. A method of quantifying promoter activations and GFP fluorescence based on luciferase activity measurements, the method comprising the step of providing the polypeptide according to claim 1.

18. A method of making a monoclonal antibody which immunoreacts with a polypeptide having both luciferase and GFP activities, the method comprising the steps of:

(a) administering to a host a polypeptide having both luciferase and GFP activities in an amount sufficient to induce the production of antibodies to the polypeptide;

(b) recovering the antibody-producing cells from the host;

(c) forming cell hybrids by fusing the antibody-producing cell to cells capable of substantially unlimited reproduction;

(d) culturing the hybrids; and

(e) collecting the monoclonal antibodies as a product of the hybrids.

19. A method of monitoring gene expression quantitatively and qualitatively in a cell using a gene fusion construct coding for a polypeptide having both luciferase and GFP activities, the method comprising the steps of:

(a) providing a gene fusion construct coding for a polypeptide having both Renilla luciferase and GFP activity;

(b) introducing the gene fusion construct into the cell;

(c) maintaining the cell containing the gene fusion construct in a manner allowing the cell to express the polypeptide; and

(d) measuring the cell for luciferase and fluorescent activity.

20. The method of claim 19, where the step of providing comprises providing a construct including a polynucleotide sequence as set forth in SEQ ID NO:1.

21. A method of monitoring gene expression quantitatively and qualitatively in a cell using a gene fusion construct coding for a polypeptide having both luciferase and GFP activities, the method comprising the steps of:

(a) providing a gene fusion construct comprising the protein of claim 1;

(b) introducing the gene fusion construct into the cell;

(c) maintaining the cell containing the gene fusion construct in a manner allowing the cell to express the polypeptide; and

(d) measuring the cell for luciferase and fluorescent activity.

FIG. 1

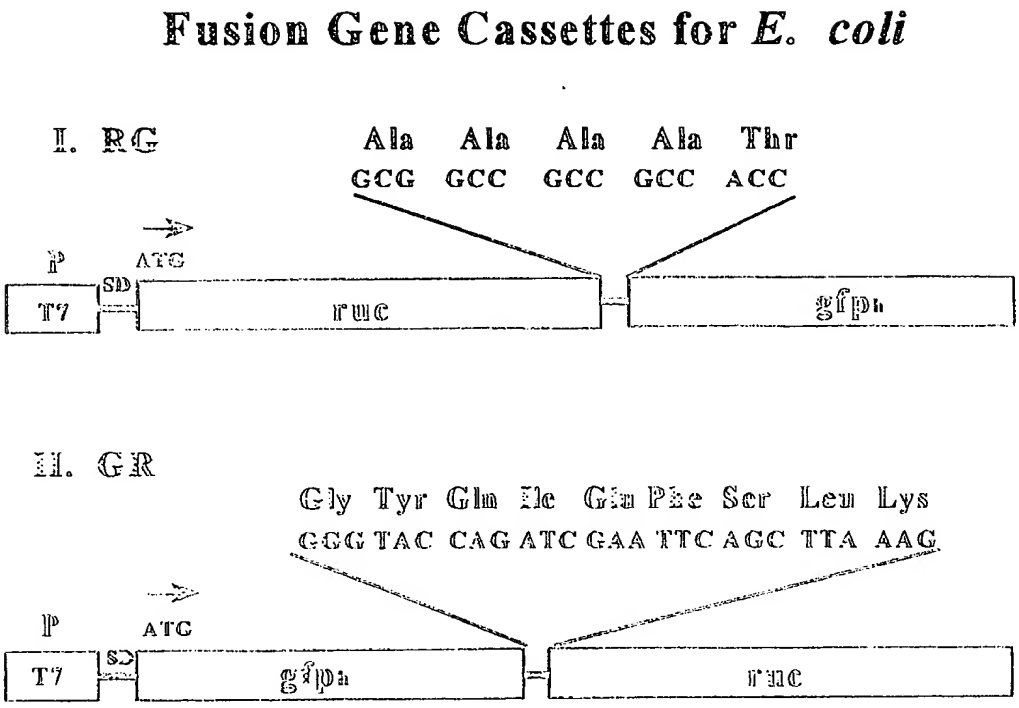

Fusion Gene Cassettes for E. coli
I. RG
Ala Ala Ala Ala Thr
GCG GCC GCC GCC ACC
P
ATG
SD
T7
rnc
gfpa
II. GR
Gly Tyr Gln Ile Glu Phe Ser Leu Lys
GGG TAC CAG ATC GAA TTC AGC TTA AAG
P
ATG
SD
T7
gfpa
rnc

FIG. 2

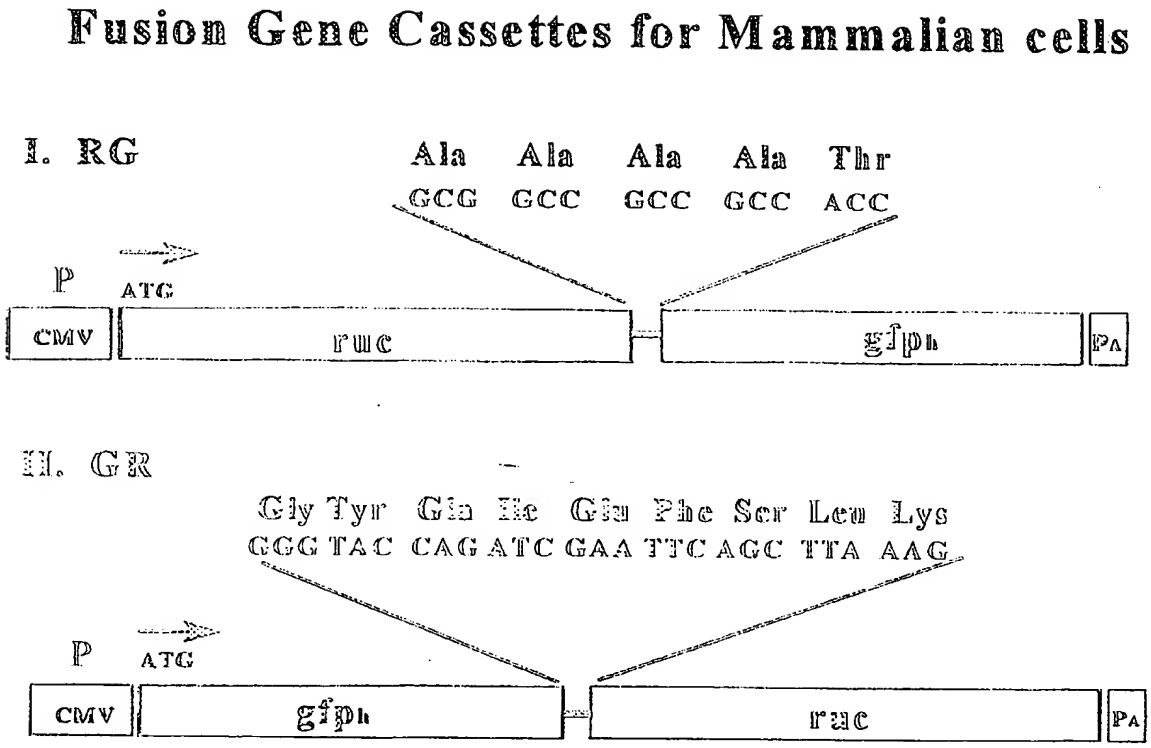
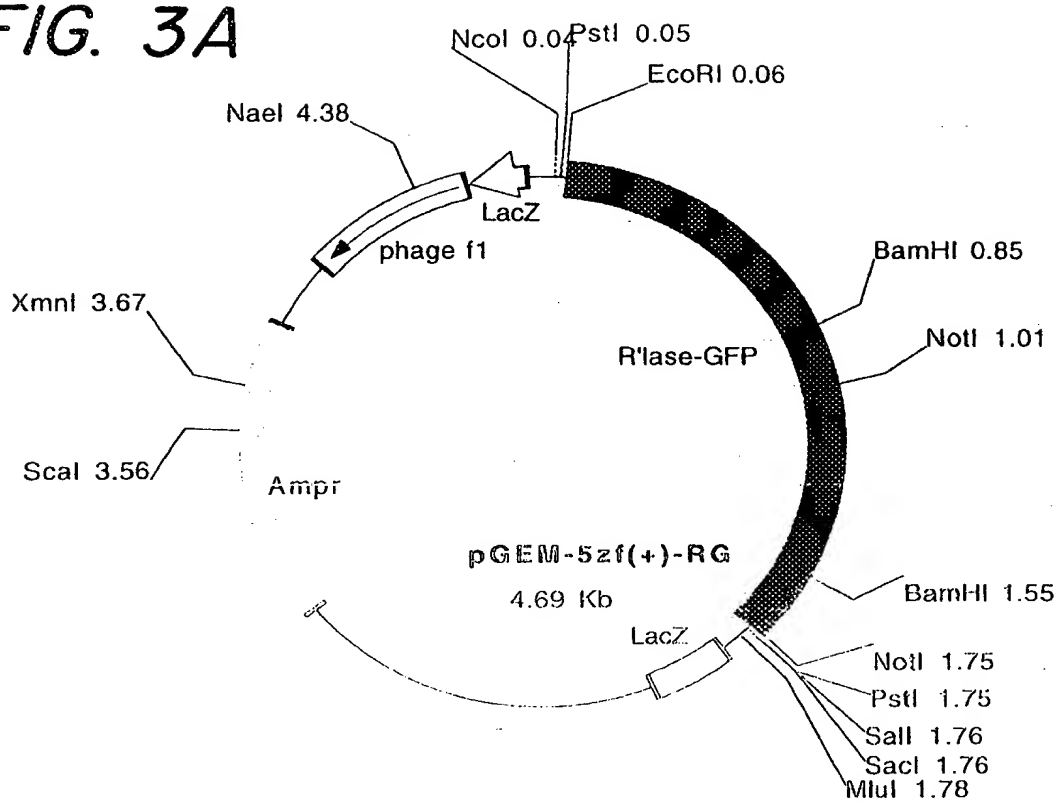

Fusion Gene Cassettes for Mammalian cells
I. RG
Ala Ala Ala Ala Thr
GCG GCC GCC GCC ACC
P
ATG
CMV
rluc
gfph
PA
II. GR
Gly Tyr Gln Ile Glu Phe Ser Leu Lys
GGG TAC CAG ATC GAA TTC AGC TTA AAG
P
ATG
CMV
gfph
rluc
PA

FIG. 3A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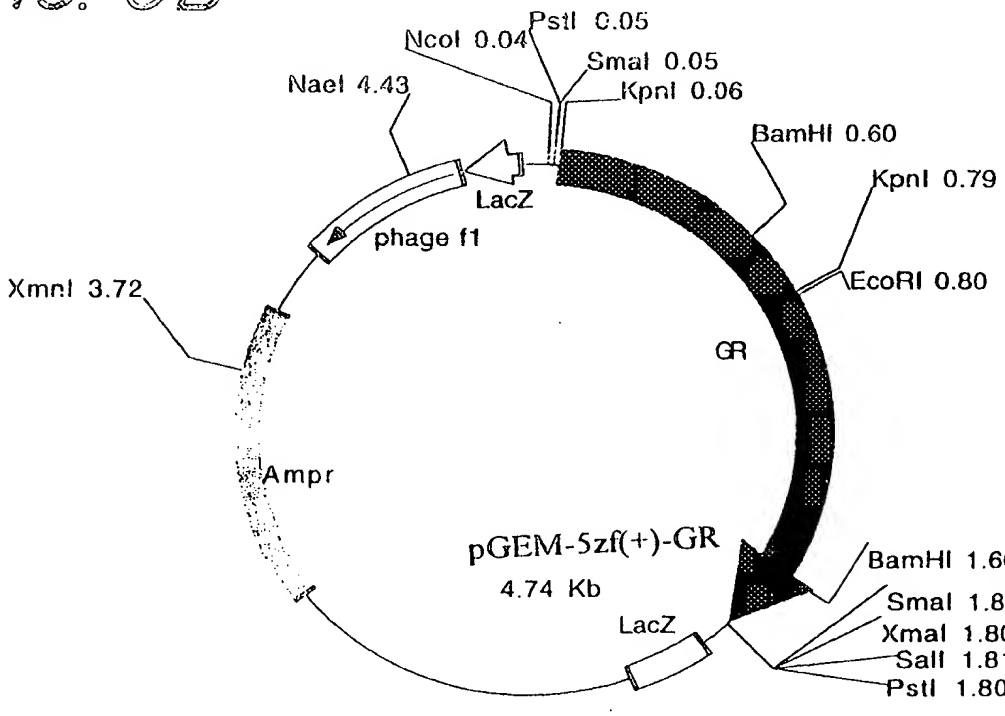
PstI 0.05
EcoRI 0.06
NaeI 4.38
LacZ
phage f1
BamHI 0.85
XmnI 3.67
NotI 1.01
R'lase-GFP
ScaI 3.56
Ampr
pGEM-5zf(+)-RG
4.69 Kb
BamHI 1.55
LacZ
NotI 1.75
PstI 1.75
SalI 1.76
SacI 1.76
MluI 1.78

FIG. 3B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PstI 0.05
SmaI 0.05
NaeI 4.43
KpnI 0.06
BamHI 0.60
KpnI 0.79
LacZ
phage f1
XmnI 3.72
EcoRI 0.80
GR
Ampr
pGEM-5zf(+)-GR
4.74 Kb
BamHI 1.60
SmaI 1.80
XmaI 1.80
LacZ
SalI 1.81
PstI 1.80

FIG. 4A

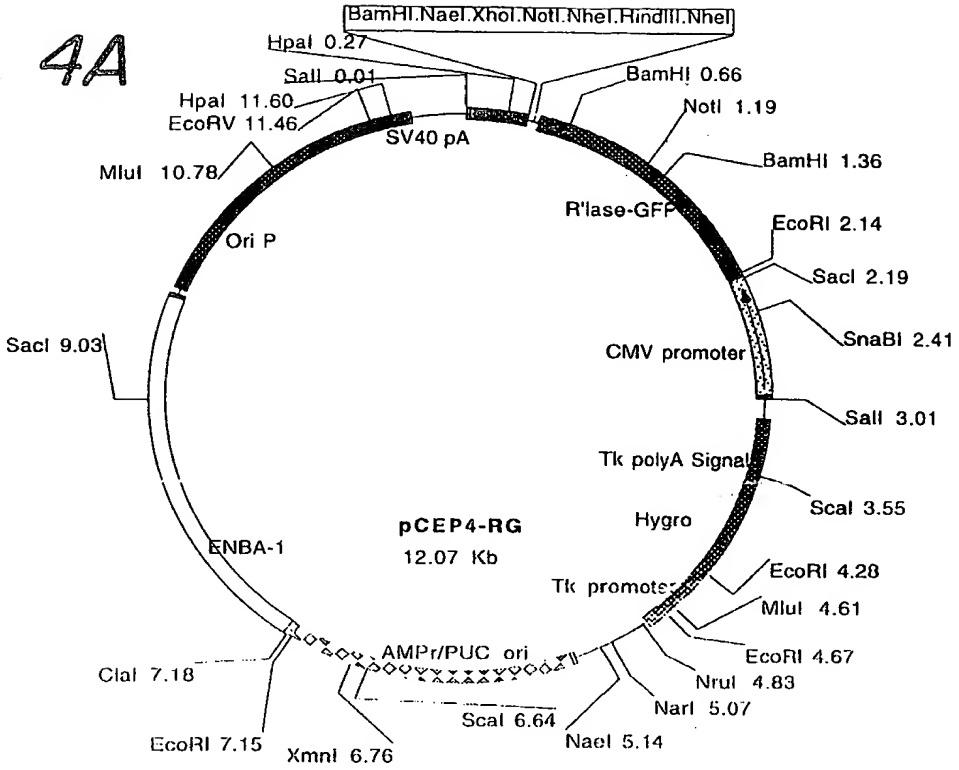

BamHI.NaeI.XhoI.NotI.NheI.HindIII.NheI
HpaI 0.27
SalI 0.01
HpaI 11.60
EcoRV 11.46
SV40 pA
BamHI 0.66
NotI 1.19
BamHI 1.36
MluI 10.78
Ori P
R'lase-GFP
EcoRI 2.14
SacI 2.19
SacI 9.03
CMV promoter
SnaBI 2.41
SalI 3.01
Tk polyA Signal
ScaI 3.55
pCEP4-RG
12.07 Kb
Hygro
ENBA-1
Tk promoter
EcoRI 4.28
MluI 4.61
AMPr/PUC ori
EcoRI 4.67
ClaI 7.18
NruI 4.83
NarI 5.07
ScaI 6.64
NaeI 5.14
EcoRI 7.15
XmnI 6.76

FIG. 4B

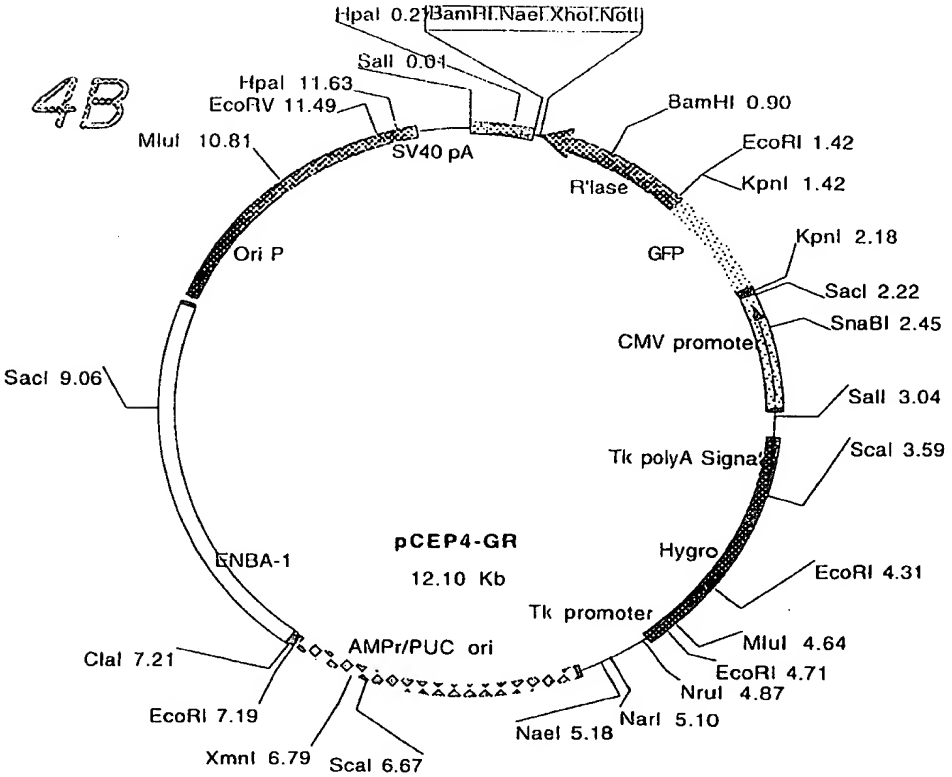

HpaI 0.21
BamHI.NaeI.XhoI.NotI
SalI 0.01
HpaI 11.63
EcoRV 11.49
SV40 pA
BamHI 0.90
MluI 10.81
EcoRI 1.42
R'lase
KpnI 1.42
Ori P
GFP
KpnI 2.18
SacI 2.22
SacI 9.06
CMV promoter
SnaBI 2.45
SalI 3.04
Tk polyA Signal
ScaI 3.59
pCEP4-GR
12.10 Kb
ENBA-1
Hygro
EcoRI 4.31
Tk promoter
MluI 4.64
ClaI 7.21
AMPr/PUC ori
EcoRI 4.71
NruI 4.87
EcoRI 7.19
NaeI 5.18
NarI 5.10
XmnI 6.79
ScaI 6.67

FIG. 5A

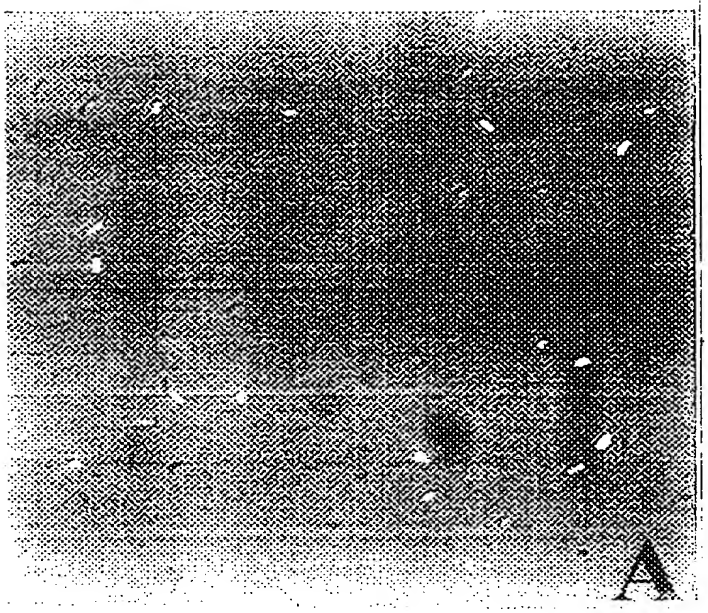


FIG. 5B

FIG. 6A

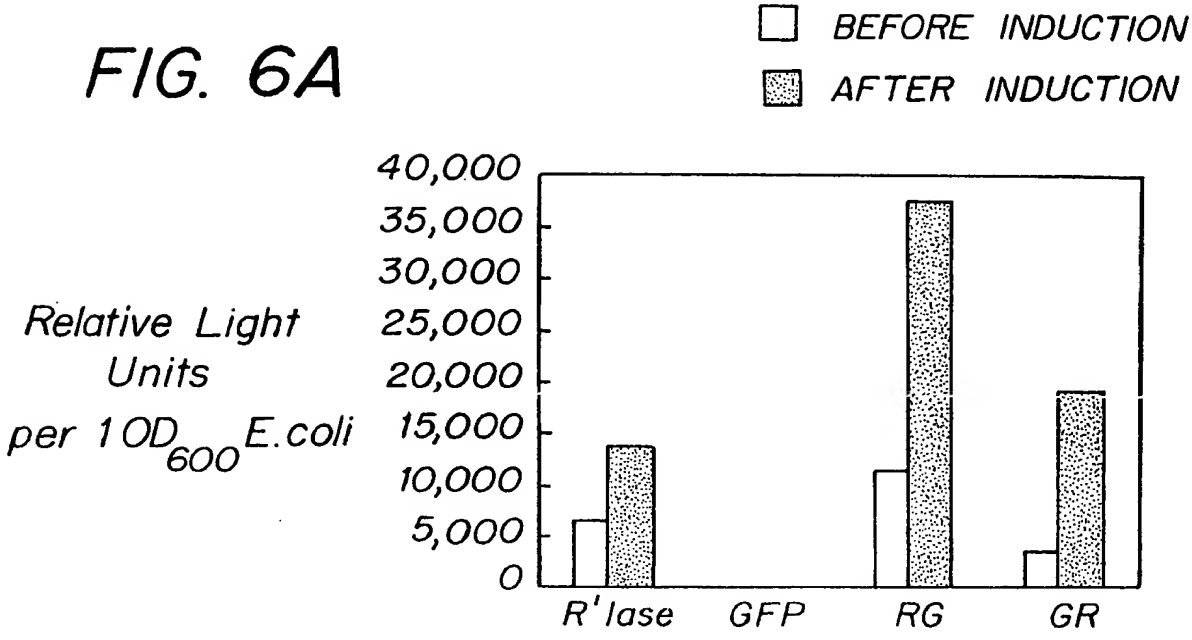

BEFORE INDUCTION
AFTER INDUCTION
Relative Light Units per 1 OD_{600} E.coli
40,000
35,000
30,000
25,000
20,000
15,000
10,000
5,000
0
R'lase
GFP
RG
GR

FIG. 6B

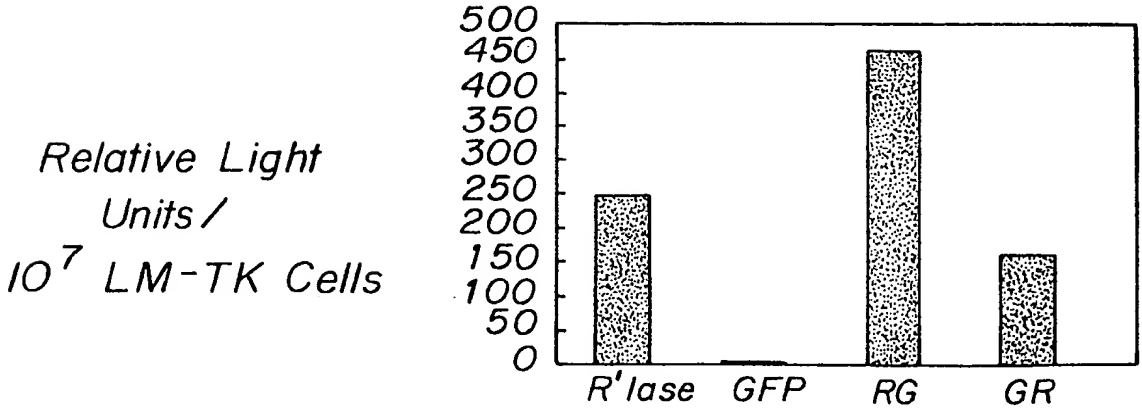

Relative Light Units / 10^7 LM-TK Cells
500
450
400
350
300
250
200
150
100
50
0
R'lase
GFP
RG
GR

FIG. 7

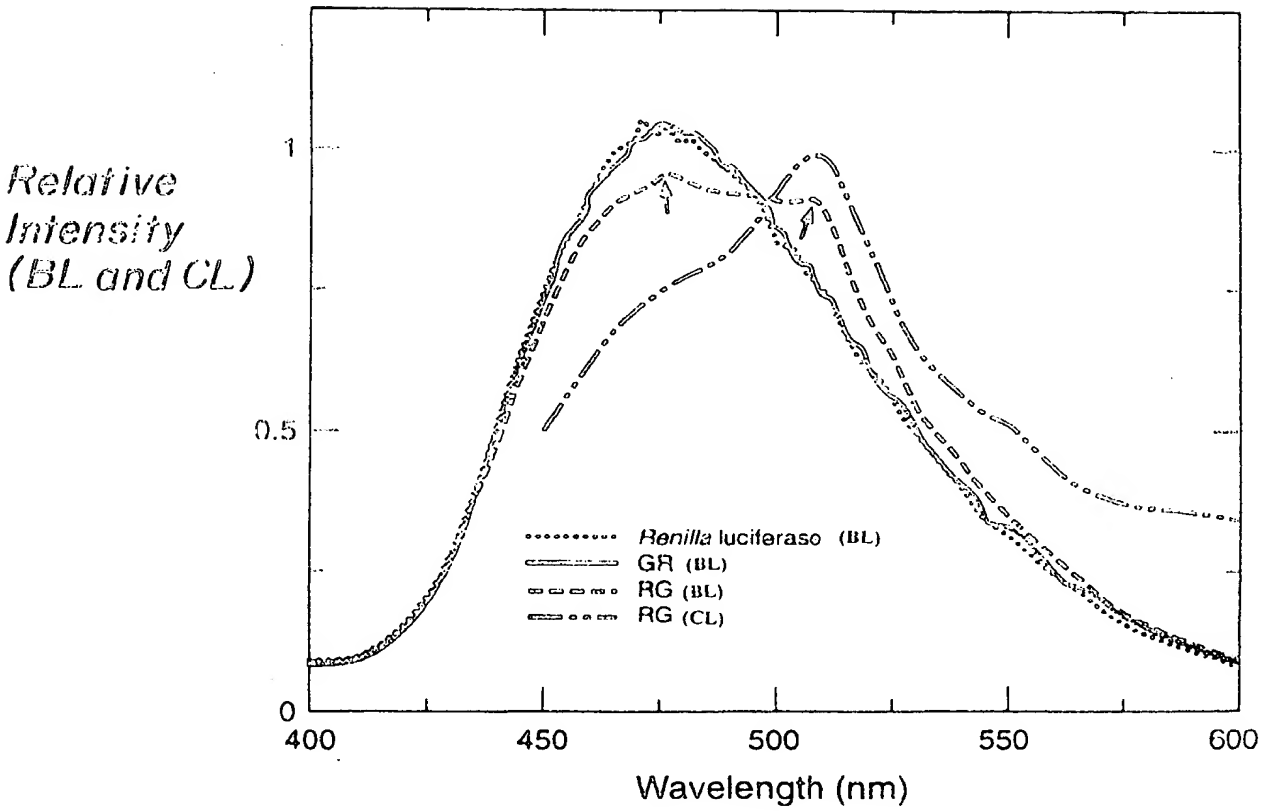

Relative Intensity (BL and CL)
1
0.5
0
400
450
500
550
600
Wavelength (nm)
Renilla luciferase (BL)
GR (BL)
RG (BL)
RG (CL)

FIG. 8

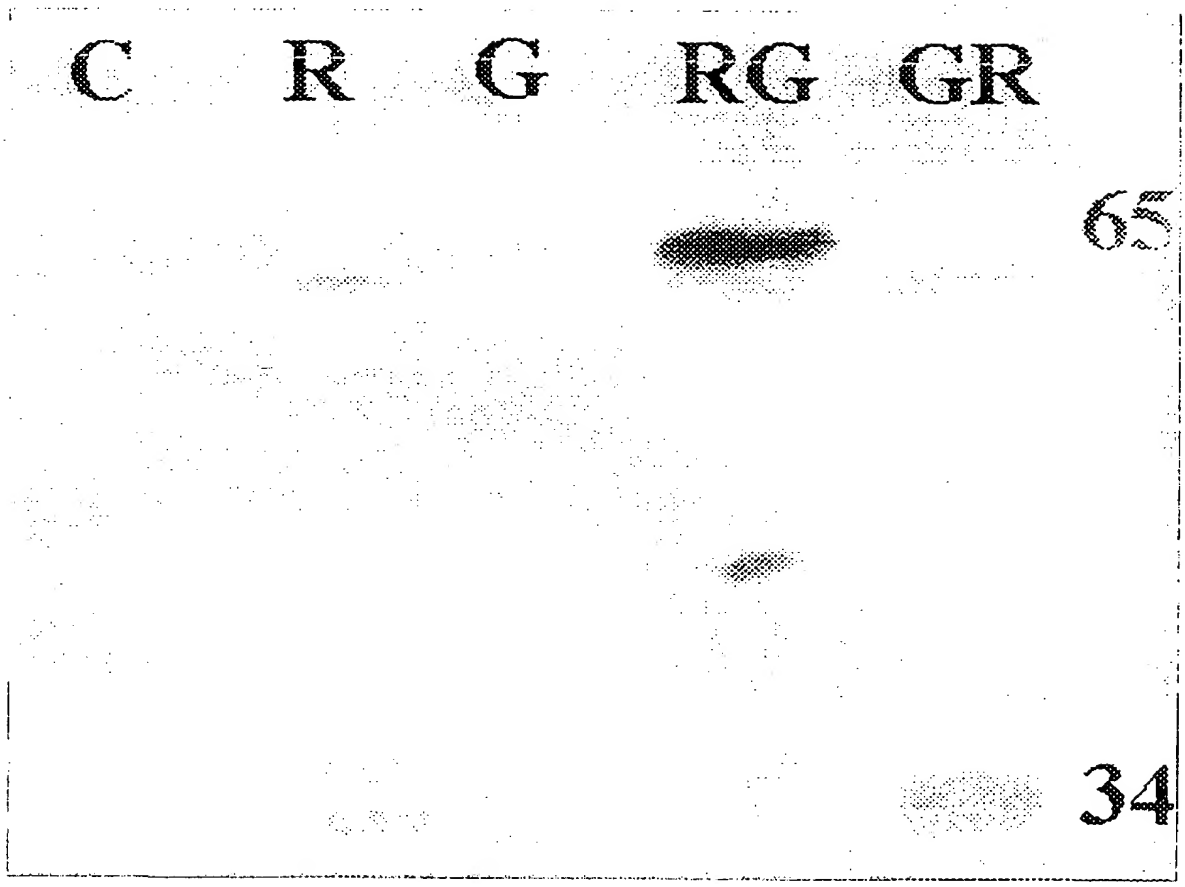


FIG. 9A

FIG. 9B

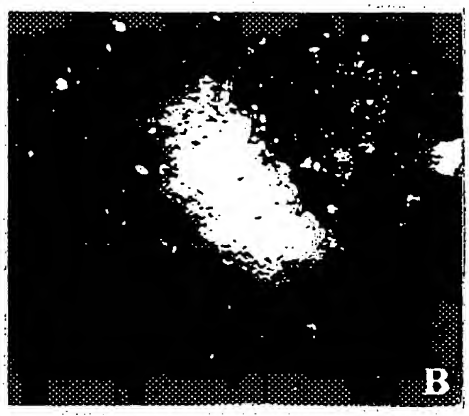


FIG. 9C

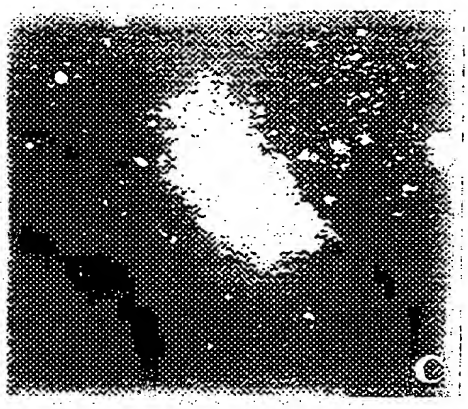


FIG. 9D

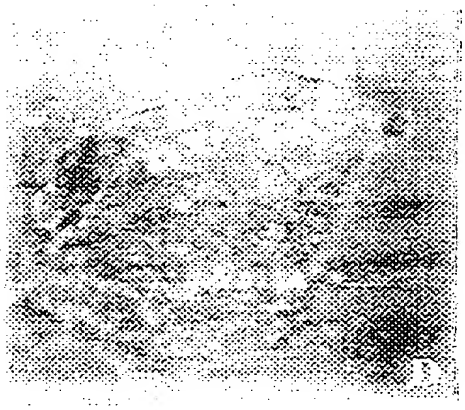


FIG. 9E

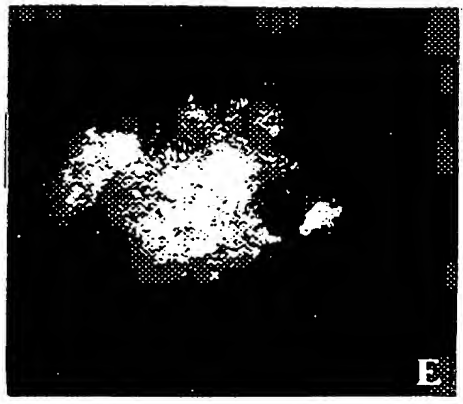


FIG. 9F

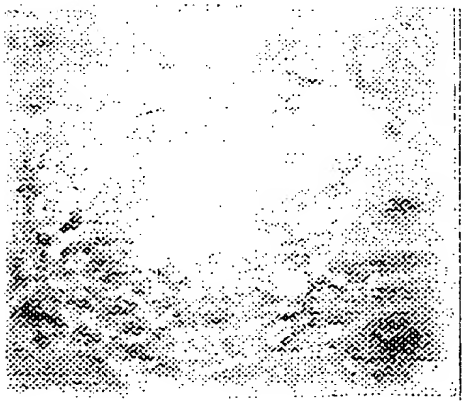


FIG. 10A

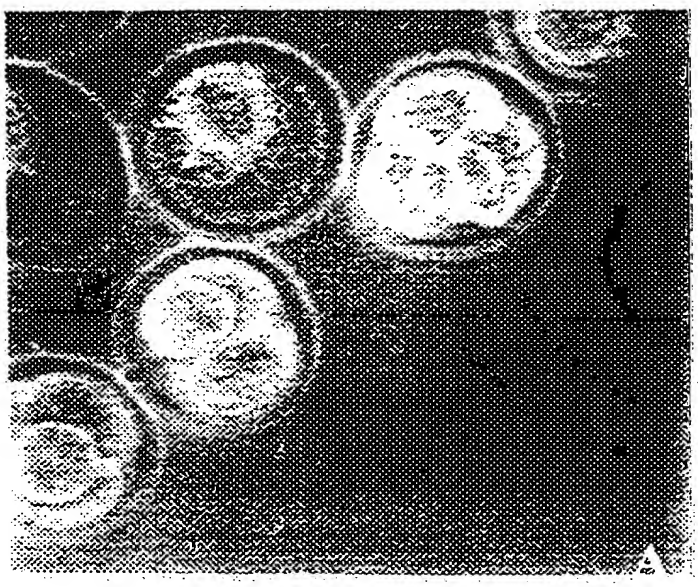
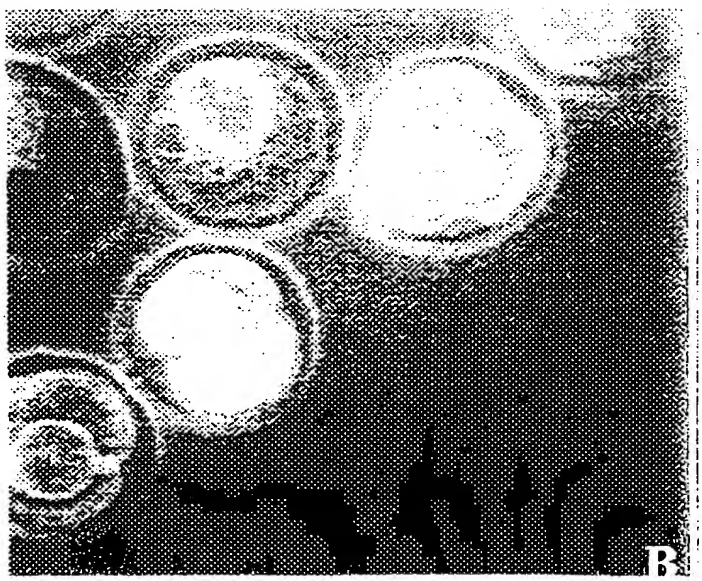


FIG. 10B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/17162

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 69.7, 189, 252.3, 320.1; 530/350, 388.1; 536/23.2, 23.4, 23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS(USPAT, EPOABS, JPOABS); STN (CAPLUS, BIOSIS)
search terms: luciferase, green fluorescent protein, renilla, aequorea, DNA, fusion, gene, antibody, monoclonal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,491,084 (CHALFIE et al) 13 February 1996, entire patent, especially column 1, lines 16-25 and claims	1,2, 6-9, 11, 13, 15-17, 19-21
A		3, 12, 14, 20
Y	US 5,292,658 (CORMIER et al) 08 MARCH 1994, entire patent, especially claims.	1, 2, 6-9, 11, 13, 15-17, 19-21
A		3, 12, 14, 20

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
11 DECEMBER 1997

Date of mailing of the international search report
23 JAN 1998

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/17162

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SANDALOVA, T. Some Notions about Structure of Bacterial Luciferase, Obtained from Analysis of Amino Acid Sequence, and Study of Monoclonal Antibody Binding. In: Biological Luminiscence, Proceedings of International School, 1st (1990), Meeting Date 1989, 330-340. Edittors: Jezowska-Trzebiatowska et al.World Science, Singapore, Singapore (Abstract)	4, 10
Y		5, 18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/17162

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/17162

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12P 21/04, 21/06; C12N 1/20, 9/02, 15/09; C07K 14/00, 16/00; C07H 21/04

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/69.1, 69.7, 189, 252.3, 320.1; 530/350, 388.1; 536/23.2, 23.4, 23.5

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-3, 6 and 7, drawn to a fusion protein having both luciferase and GFP activities.

Group II, claim(s) 4, 5 and 10, drawn to a monoclonal antibody against said fusion protein.

Group III, claim(s) 8, 9 and 11-17, drawn to a DNA encoding said fusion protein, a vector containing said DNA, a cell transformed with the same, a method of producing said fusion protein using a transformed cell and 1st method of use of said DNA.

Group IV, claim 18, drawn to a method of making a monoclonal antibody.

Group V, claim(s) 19-21, drawn to 2nd method of use of DNA encoding fusion protein.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: a fusion protein of Group I, an antibody of Group II and a DNA of Group III are different compounds with different structures, functions and utilities. Luciferase and GFP as well DNAs encoding them and gene fusion constructs based on each of them are known in the prior art. An antibody against both proteins are known. Therefore, a fusion protein containing either luciferase or GFP lacks a special technical feature with a DNA encoding thereof and an antibody against it.

Inventions of Groups IV and V are drawn to materially different methods. Method of Group IV employs immunization of an animal with a fusion protein and a hybridoma production, whereas a method of Group V employs a DNA construct encoding a fusion protein.

PCT Rule 1.475(d) does not provide for multiple products or methods within a single application and therefore, unity of invention is lacking with regard to Groups I-V.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: A METHOD TO ENABLE ASSESSMENT OF GROWTH AND DEATH OF MICRO-ORGANISMS

(57) Abstract: A method to enable the assessment of the growth rate and death rate of a micro-organism within a chosen time period in an environment of interest. The method is characterised in that: a) two reporter genes are introduced to said micro-organism wherein, the reporter genes used code for luminescent and/or fluorescent products and at least two of the following products: an essentially stable product produced in an essentially known proportion to the total amount of cells of said micro-organism that are or have been alive within said chosen time period; a product present in an essentially known proportion to the amount of cells alive at any moment within said chosen time period; and an essentially stable product produced in an essentially known proportion to the total amount of cells of the said micro-organism that have died within said chosen time period, and said products can be measured through their luminescence and/or fluorescence; b) the said micro-organism is incubated and said luminescence and/or fluorescence is detected after said chosen time periods, and c) the growth and death rate of the said micro-organism is assessed based on at least two of the following: the known proportion of luminescence or fluorescence to the amount of cells alive after any said chosen time period; the known proportion of luminescence or fluorescence to the total amount of cells that are or have been alive within any said chosen time period; and the known proportion of luminescence or fluorescence to the total amount of cells that have died within any said chosen time period.

WO 00/75367 A1

A METHOD TO ENABLE ASSESSMENT OF GROWTH AND DEATH OF MICRO-ORGANISMS

This invention relates to a method to enable the assessment of growth and death of a micro-organism within a chosen time period in an environment of interest.

BACKGROUND OF THE INVENTION

When studying growth and death of a micro-organism under the influence of specific environments, e.g. production and storage environments that e.g. could or could not be refrigerated, or involving chemicals or matrixes, e.g. antibiotics, microbial toxins, heavy metals and serum complement, microbial cultures are most often incubated for hours or days. In these circumstances death and growth occur simultaneously. If additionally some of the cells lyse, e.g. when analysing the serum complement, it is difficult to know to what one should compare the amount of living cells at the end of the experiment. Convenient methods to determine the number of living cells, e.g. by measuring luciferase bioluminescence, are known but if no more information is available it is impossible to assess to what extent growth or/and death of the micro-organisms takes or has taken place.

Growth rates and death rates of micro-organisms in specific environments are of interest in many areas. Death rates and growth rates of micro-organisms and especially harmful and/or pathogenic micro-organisms are of importance in risk assessments of products of the pharmaceutical industry and products for human consumption with regard to their production, storage and distribution to the consumers. Knowledge of death and growth rates of micro-organisms are of particular importance in specific applications such as in the development of antibiotics, disinfectants and bactericidal products or monitoring of sterilisation, disinfection and cleaning processes.

Reporter genes coding for luminescent or/and fluorescent products have been introduced to micro-organisms to enable the assessment of the quantity or survival of living micro-organisms (WO 96/23898, WO 98/14605, WO 98/30715, WO 98/36081, US 5,824,468). Even simultaneous use of luminescent and fluorescent markers has been used (Fratamico et al., Journal of Food Protection, Vol 50 No 10, 1997, 1167–1173). Luminescent and fluorescent markers have, however, only been used as markers for survival of micro-organisms and the use of two different markers within one micro-organism enabling the differentiation between growth and death rates has not been reported.

OBJECT AND SUMMARY OF THE INVENTION

The object of the present invention is to provide a method to enable the assessment of the growth and death of a micro-organism within a chosen time period in an environment of interest by introducing into said micro-organism at least two reporter genes. The method is characterised in that

a) said reporter genes code for luminescent and/or fluorescent products and within said time period and environment at least two said products of the following are produced:

   i) an essentially stable product produced in a, within the environment of interest, essentially known proportion to the total amount of cells of said micro-organism that are or have been alive within said chosen time period,

   ii) a product present in said environment of interest in an essentially known proportion to the amount of cells alive at any moment within said chosen time period and

iii) an essentially stable product produced in a, within the environment of interest, essentially known proportion to the total amount of cells of said micro-organism that have died within said chosen time period,

and said products can be measured through their luminescence and/or fluorescence;

b) said micro-organism is incubated within the environment of interest and said luminescence and/or fluorescence is detected after said chosen time period, and

c) the growth and death rate of the said micro-organism is assessed based on at least two of the following:

i) the known proportion of luminescence or fluorescence to the amount of cells alive after any said chosen time period,

ii) the known proportion of luminescence or fluorescence to the total amount of cells that are or have been alive within any said chosen time period, and

iii) the known proportion of luminescence or fluorescence to the total amount of cells that have died within any said chosen time period.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows plasmid pGFP+luc* including genes for both GFP and firefly luciferase.

Figure 2 shows fluorescence during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of ethanol in the cell culture.

Figure 3 shows luminescence during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of ethanol in the cell culture.

Figure 4 shows the amount of living cells, i.e. colony forming units, according to plating during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of ethanol in the cell culture.

Figure 5 shows the percentage of living cells according to live/dead staining and flow cytometric analysis during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of ethanol in the cell culture.

Figure 6 shows fluorescence before and after incubation with serum complement during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of serum complement in the cell culture.

Figure 7 shows luminescence before and after incubation with serum complement during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of serum complement in the cell culture.

Figure 8 shows the percentage of living cells according to plating during growth phase of *E. coli* with plasmid pGFP+luc* at 30 °C as a function of the concentration of serum complement in the cell culture.

DETAILED DESCRIPTION OF THE INVENTION

The method according to the present invention can be used to assess the growth and death rate of a micro-organism within a chosen time period in any particular environment of interest. The method is applicable if two different marker genes can be introduced to the micro-organism that code for luminescent and/or fluorescent products, and the products of these fulfil at least two of the following three criteria:

a) a said luminescent product luminesces or said fluorescent product fluoresces in an essentially known proportion to the amount of cells of said micro-organism alive within said chosen time period ;

b) a said luminescent product luminesces or said fluorescent product fluoresces in

an essentially known proportion to the amount of cells of said micro-organism that are or have been alive within said chosen time period, and

c) a said luminescent product luminesces or said fluorescent product fluoresces in an essentially known proportion to the amount of cells of said micro-organism that have died within said chosen time period.

In the present application the concept "micro-organism" means any micro-organism into which marker genes can be introduced so, that they will function according to the invention. "Micro-organism" can therefore stand for bacteria, yeast or fungi.

The concept of "introducing a marker gene into a micro-organism" means any method by which a marker gene can be made to function within the micro-organism according to the invention. One way of introducing marker genes into micro-organism is by constructing a recombinant strain. This can be done by transforming a strain with a plasmid including the marker genes. An alternative way to introduce reporter genes to bacteria is to utilise transposable elements. In this technique, reporter genes are inserted between insertion sequences in a delivery plasmid. The plasmid is then introduced to a cell by e.g. conjugation of transformation, and once inside the cell, genes surrounded by the insertion sequences are integrated into bacterial chromosome. Integration is stable, i.e. there is no need for a selectable marker such as antibiotic resistance.

Assessment of the growth rate and death rate of a micro-organism can be of interest in many specific environments. Within pharmaceutical research the effect of different drugs and candidates for drugs, e.g. antibiotics, on the survival of pathogenic, but also the beneficial micro-organisms of the gut, is of interest. Thus the ultimate interest is in the behaviour of these micro-organisms in a physiological environment affected by drugs.

Another vast area where the possibility of assessing growth and death rate of specific micro-organisms is of interest is that of production, processing, storage and

distribution of all products for human consumption. In this area the behaviour of pathogenic or potentially harmful micro-organisms in the different environments of the life cycle of these products is of special interest and involves many different aspects such as the influence of temperature, humidity or light and the possible use of preservatives etc.

Additionally growth and death rates of micro-organisms can be of interest for environmental evaluations e.g. when evaluating the effect of emissions into the environment.

Luminescent or fluorescent products coded by reporter genes in different embodiments of this invention can vary as long as their proportion to either the total amount of cells alive, to cells that are or have been alive, or to cells that have died is essentially known. Growth and death rate can be assessed if two of the following: cells alive, cells that are or have been alive, or cells that have died can be determined. Thus luminescence and/or fluorescence measured can be e.g. of a product which is expressed e.g. constitutively or triggered by a specific phase (e.g. replication or death) of the lifecycle of each cell, is stable or labile or which luminescence or fluorescence is dependant on a factor that relates e.g. to a specific phase of the lifecycle of each cell. Depending on the individual characteristics of said product—how produced, stable or labile, possible dependence of its luminescence or fluorescence of said factors etc.— the measured luminescence or fluorescence can be in proportion to one of the three unknown of which two must be known to be able to assess the growth rate and death rate of said cells.

According to one specific embodiment of the invention assessment of the growth and death rate of an *Esherichia coli* strain under the influence of different chemicals or matrixes was enabled by constructing a recombinant strain, which expresses both luciferase and GFP. Altogether the effect of a number of different chemicals and matrixes, such as $CdCl_2$, ethanol, the antibiotics chloramphenicol, rifampicin, and

tetracyclin, as well as serum complement on said recombinant *E. coli* strain was tested and found applicable.

The invention will be described in more detail by the following study in which the growth rate and death rate of a recombinant *Esherichia coli* strain, which expresses both luciferase and GFP, is assessed under the influence of ethanol or serum complement.

Summary of the study

Genes for luciferase and green fluorescent protein have recently raised interest as reporter genes. Luciferase is an enzyme that produces luminescence in the presence of substrate luciferin, molecular oxygen and ATP. Green fluorescent protein (GFP), produces green fluorescence when excited with light. Many mutated forms of GFP have been introduced: some have different excitation and emission wavelengths from the wild type and some mutants form more stable proteins at higher temperatures.

We constructed a recombinant strain of *E. coli*, which expresses both luciferase and GFP. In our construction we used a mutant of GFP, which is more stable at temperatures over +30 °C and it matures quicker than the wild type. Luciferase was from North American firefly, *Photinus pyralis*.

The *E. coli* strain MC1061 was transformed with a plasmid including genes for both GFP and firefly luciferase. Figure 1 describes the plasmid in general. The sequence of the plasmid is disclosed in the sequence listing. Essential codings of the sequence are as follows:

lac promoter	95–199
GFP	289–1008
firefly luciferase	1044–2696
β-lactamase	3251–4111

In our construct, see Figure 1, the luciferase gene is situated next to the GFP gene and both genes are transcribed in the same direction. The transcription is started at the lac promoter in front of GFP. The lac promoter is constitutively active, because the MC1061 cells lack its repressor. The plasmid also has a gene for ampicillin resistance (β-lactamase).

The transformed *E. coli* strain was propagated under the influence of different concentrations of ethanol or serum complement.

Methods

Growth conditions

One colony from a pure culture plate was inoculated in 5 ml of LB-medium with ampicillin (100 µg/ml) and grown at +37 °C in a shaker, 250 rpm, for about three hours. After that, the number of cells per millilitre was determined with flow cytometry by using fluorescent spherical latex particles as a reference. One million cells were then removed to an erlenmeyer with 50 ml of LB medium and ampicillin. The culture was grown over night in a shaker, 190 rpm, at room temperature to prevent the culture from growing into the stationary phase during the night. In the morning, the culture was transferred to and grown in a shaker, 330 rpm, until the stationary phase was reached or used after growth at +30 °C for about 1 h to study the influence of ethanol or serum complement as described below.

Influence of chemicals on the propagation of *E. coli*

The culture obtained as described above was used to study the influence of ethanol or serum complement as follows:

Ethanol

Ethanol (Aa, Primalco Oy) was diluted into pure water to obtain concentrations of 50, 45, 25, 10, 5, 1 and 0 % of ethanol when 500 µl of said dilution was added to 500 µl of said culture in an eppendorf tube. The mixture was vortexed and incubated for 5 minutes before measuring fluorescence and luminescence. Live cells were again counted by plating and also by live/dead staining. In the live/dead protocol used the stain *cyto 9* stains all cells whereas propidium iodide stains only the dead cells. After staining, cells are passed through a flow cytometer, with which dead and live cells can be differentiated and their proportion determined. (Virta et al. (1998) Appl. Environ. Microbiol. 64: 515–519.)

Serum complement

The influence of serum complement on the said recombinant *E. coli* strain was studied using an incubation time of 90 min as described for a different recombinant *E. coli* strain used in Virta et al. (1998) Appl. Environ. Microbiol. 64: 515–519.

Fluorescence and luminescence measurements

The measurements were done with a combined fluoro- and luminometer, Fluoroscan Ascent FL, provided by Labsystems Ltd. (Helsinki, Finland). Cell growth was simultaneously followed with a flow cytometer.

For the measurements, 100 µl of bacterial culture was pipetted into the microtiter plate wells. Fluorescence was measured using 485 nm for excitation and 510 nm for emission. Measuring time was 20 ms. After the fluorescence measurement 100 µl of luciferin in 0.1 M citric acid-sodium citrate buffer (pH 5.0) was dispensed into the wells and the plate was shaken for two minutes (shaking diameter 1 mm, 1 020 rpm), after which luminescence was recorded with a measuring time of 1000 ms.

Plating

Samples for plating were diluted 10^2 to 10^7 fold with 150 mM NaCl and plated onto L agar plates (L broth containing 1.6 % agar). Colonies were counted after overnight incubation at 37 °C.

Live/dead staining and Flow cytometric analysis

Bacteria from 1 000 µl of cell culture were used for live/dead staining and flow cytometric analysis using a LIVE/DEAD BacLight bacterial viability kit (catalogue no. L-7005) for microscopy and quantitative analysis obtained from Molecular Probes Europe (Leiden, The Netherlands) and Fluoresbrite beads (diameter, 1.8 µm) obtained from Polysciences Inc. (Warrington, Pa.) as described in Virta et al. (1998) Appl. Environ. Microbiol. 64: 515–519.

Results

When the cultures were transferred to +30 °C, the cells grew logarithmically for 1—4 hours depending on the initial cell concentration. Luminescence and fluorescence rose logarithmically and were essentially constant per cell. Thus cell number could be assessed based on luminescence or fluorescence.

When ethanol was added in different concentrations to the growth medium (see Figures 4 and 5) death was, after a very short incubation period of 5 min, more or less unsignificant at ethanol concentrations below 5 % and became more significant with increasing ethanol concentration reaching very pronounced significance at ethanol concentrations above 10 %. Correspondingly fluorescence (Figure 2) was essentially constant whatever the ethanol concentration in spite of dramatically decreasing corresponding live cell count according to plate count (Figure 4) and percentage of live cells according to the live/dead staining (Figure 5) whereas luminescence (Figure 3) dropped dramatically essentially corresponding to the

dramatic drop in plate count (Figure 4) and the percentage of live cells (Figure 5) with increased ethanol concentration.

The effect of serum complement on the growth and death of *E. coli* is shown in Figures 6 to 8. Fluorescence (Figure 6) and luminescence (Figure 7) are shown before (squares) and after (circles) incubation for 90 minutes with serum complement. Fluorescence (Figure 6) is slightly increased, during incubation regardless of the concentration of serum, whereas luminescence (Figure 7) decreases during incubation with increasing serum concentration. The decrease of luminescence during incubation with increasing concentrations of serum correlates clearly with the percentage of cells alive after incubation (Figure 8).

CLAIMS

1. A method to enable the assessment of the growth rate and death rate of a micro-organism within a chosen time period in an environment of interest by introducing into said micro-organism at least two reporter genes, which method is **characterised** in that

a) said reporter genes code for luminescent and/or fluorescent products and within said time period and environment at least two said products of the following are produced:

i) an essentially stable product produced in a, within the environment of interest, essentially known proportion to the total amount of cells of said micro-organism that are or have been alive within said chosen time period,

ii) a product present in said environment of interest in an essentially known proportion to the amount of cells alive at any moment within said chosen time period, and

iii) an essentially stable product produced in a, within the environment of interest, essentially known proportion to the total amount of cells of said micro-organism that have died within said chosen time period,

and said products can be measured through their luminescence and/or fluorescence;

b) said micro-organism is incubated within the environment of interest and said luminescence and/or fluorescence is detected after said chosen time period, and

c) the growth and death rate of the said micro-organism is assessed based on at least two of the following:

i) the known proportion of luminescence or fluorescence to the amount of cells alive after any said chosen time period,

ii) the known proportion of luminescence or fluorescence to the total amount of cells that are or have been alive within any said chosen time period, and

iii) the known proportion of luminescence or fluorescence to the total amount of cells that have died within any said chosen time period.

2. The method according to claim 1 **characterised** in that said micro-organism is a gram negative bacteria, e.g. *Escherichia coli*.

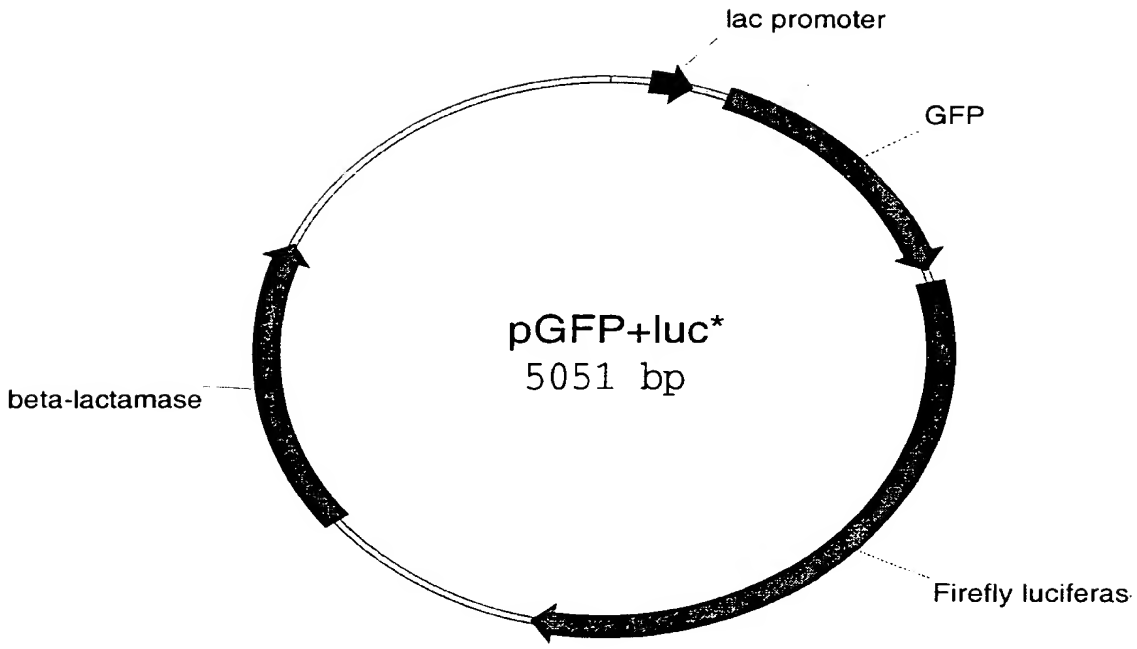
3. The method according to claim 1 or 2 **characterised** in that

a) one reporter gene coding for a luminescent product is luciferase, which is used for the determination of amount of cells alive at any moment within said chosen time period, and

b) another reporter gene coding for a fluorescent product is green fluorescent protein (GFP), which is used for the determination of total amount of cells of said micro-organism that are or have been alive within said chosen time period.

4. The method according to claim 1 or 2 **characterised** in that said reporter genes are introduced into said micro-organism in a plasmid.

5. A method according to the methods of claim 3 and 4 **characterised** in that said plasmid is pGFP+luc* (SEQ ID NO: 1).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GFP
pGFP+luc*
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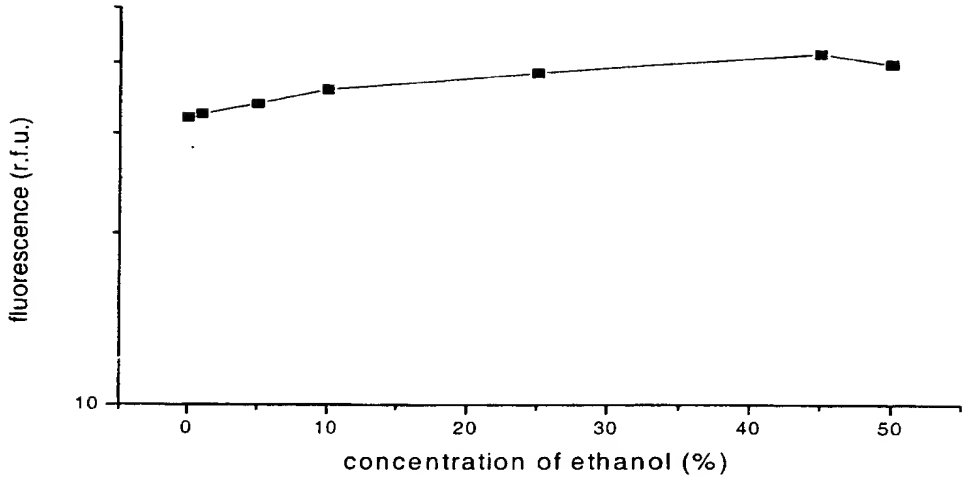
Firefly luciferas

Figure 1



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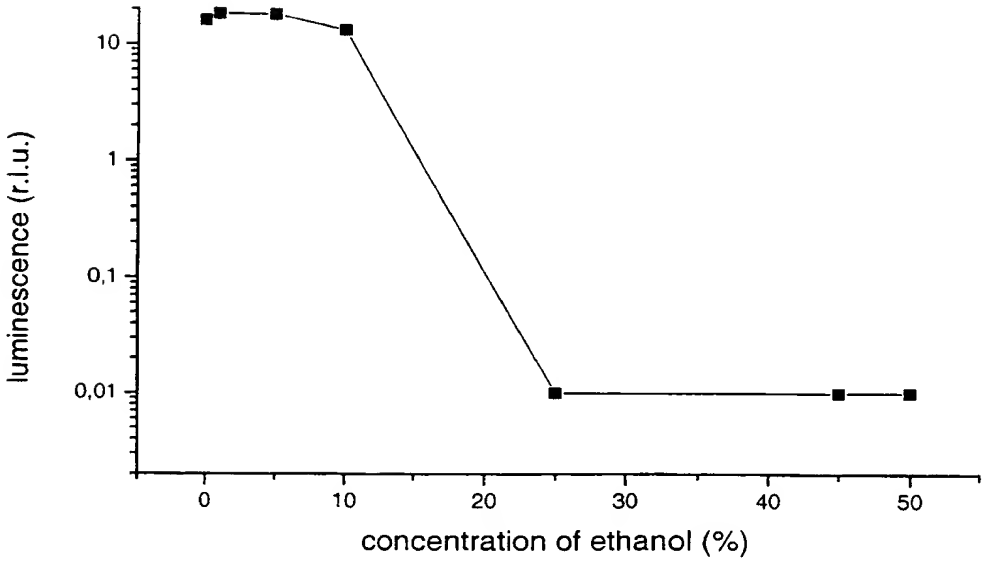

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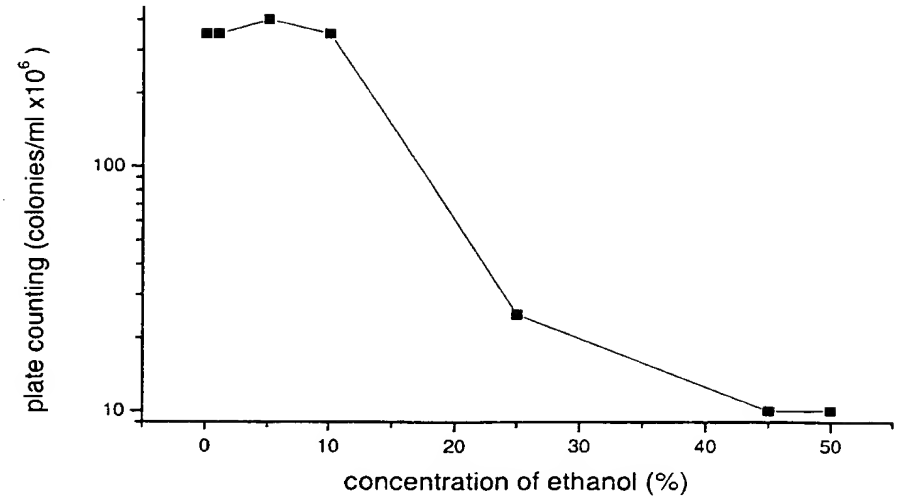

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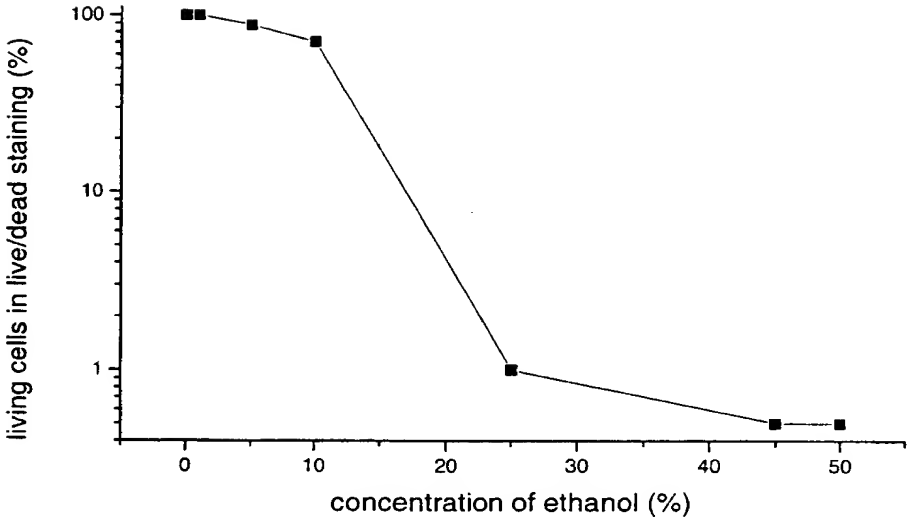

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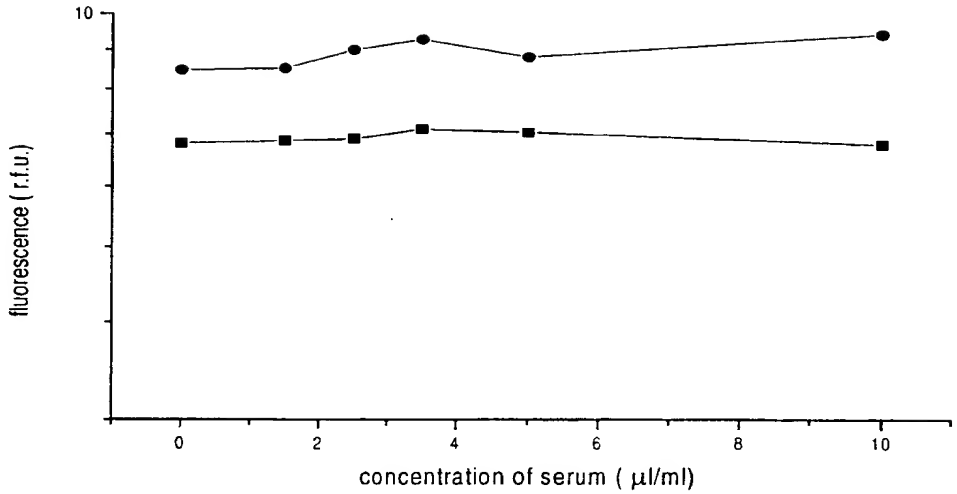

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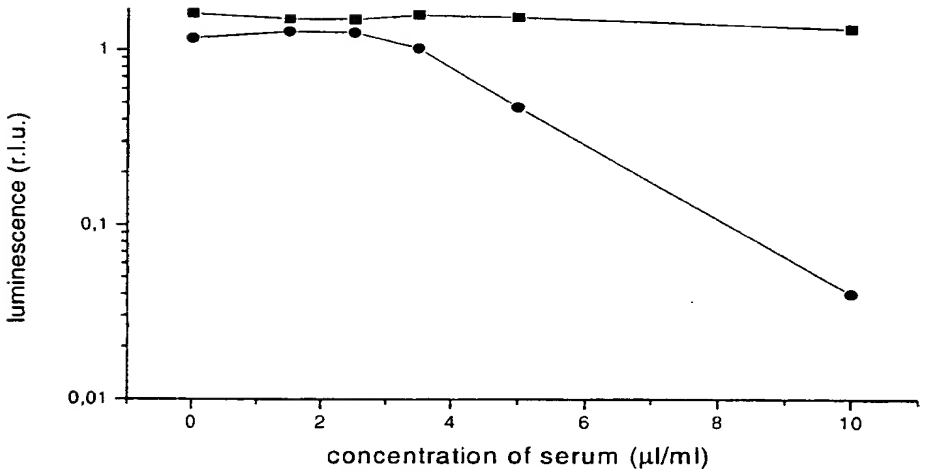

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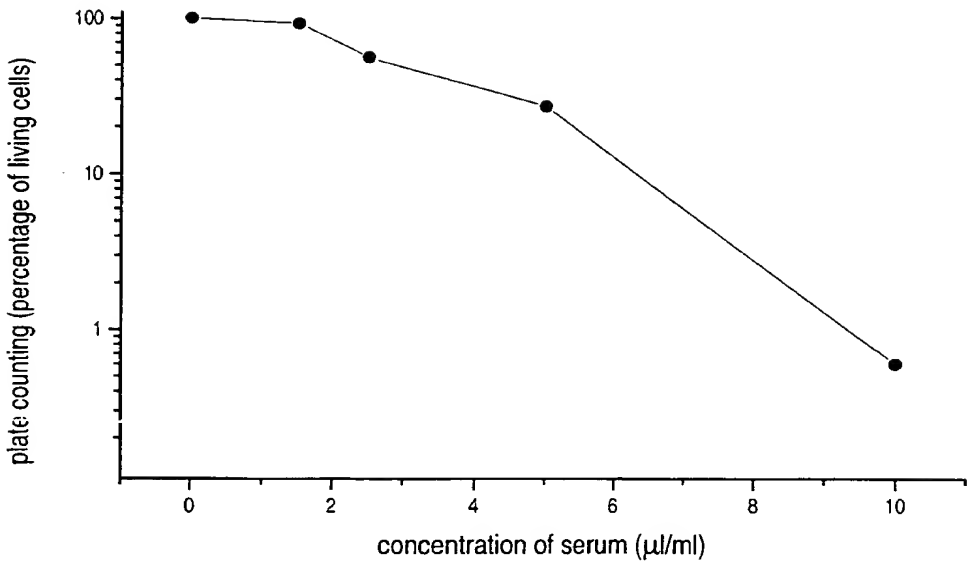
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concentration of serum (µl/ml)

Figure 8

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Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
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Val Asn Ile Thr Tyr Ala Glu Tyr Phe Glu Met Ser Val Arg Leu Ala
50 55 60
Glu Ala Met Lys Arg Tyr Gly Leu Asn Thr Asn His Arg Ile Val Val
65 70 75 80
Cys Ser Glu Asn Ser Leu Gln Phe Phe Met Pro Val Leu Gly Ala Leu
85 90 95
Phe Ile Gly Val Ala Val Ala Pro Ala Asn Asp Ile Tyr Asn Glu Arg
100 105 110
Glu Leu Leu Asn Ser Met Asn Ile Ser Gln Pro Thr Val Val Phe Val
115 120 125
Ser Lys Lys Gly Leu Gln Lys Ile Leu Asn Val Gln Lys Lys Leu Pro
130 135 140
Ile Ile Gln Lys Ile Ile Ile Met Asp Ser Lys Thr Asp Tyr Gln Gly
145 150 155 160
Phe Gln Ser Met Tyr Thr Phe Val Thr Ser His Leu Pro Pro Gly Phe
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180 185 190

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        195                 200                 205
Ala Leu Pro His Arg Thr Ala Cys Val Arg Phe Ser His Ala Arg Asp
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Pro Ile Phe Gly Asn Gln Ile Ile Pro Asp Thr Ala Ile Leu Ser Val
225                 230                 235                 240
Val Pro Phe His His Gly Phe Gly Met Phe Thr Thr Leu Gly Tyr Leu
                245                 250                 255
Ile Cys Gly Phe Arg Val Val Leu Met Tyr Arg Phe Glu Glu Glu Leu
            260                 265                 270
Phe Leu Arg Ser Leu Gln Asp Tyr Lys Ile Gln Ser Ala Leu Leu Val
        275                 280                 285
Pro Thr Leu Phe Ser Phe Phe Ala Lys Ser Thr Leu Ile Asp Lys Tyr
    290                 295                 300
Asp Leu Ser Asn Leu His Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser
305                 310                 315                 320
Lys Glu Val Gly Glu Ala Val Ala Lys Arg Phe His Leu Pro Gly Ile
                325                 330                 335
Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Ile Leu Ile Thr
            340                 345                 350
Pro Glu Gly Asp Asp Lys Pro Gly Ala Val Gly Lys Val Val Pro Phe
        355                 360                 365
Phe Glu Ala Lys Val Val Asp Leu Asp Thr Gly Lys Thr Leu Gly Val
    370                 375                 380
Asn Gln Arg Gly Glu Leu Cys Val Arg Gly Pro Met Ile Met Ser Gly
385                 390                 395                 400
Tyr Val Asn Asn Pro Glu Ala Thr Asn Ala Leu Ile Asp Lys Asp Gly
                405                 410                 415
Trp Leu His Ser Gly Asp Ile Ala Tyr Trp Asp Glu Asp Glu His Phe
            420                 425                 430
Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln
        435                 440                 445
Val Ala Pro Ala Glu Leu Glu Ser Ile Leu Leu Gln His Pro Asn Ile
    450                 455                 460
Phe Asp Ala Gly Val Ala Gly Leu Pro Asp Asp Asp Ala Gly Glu Leu
465                 470                 475                 480
Pro Ala Ala Val Val Val Leu Glu His Gly Lys Thr Met Thr Glu Lys
                485                 490                 495
Glu Ile Val Asp Tyr Val Ala Ser Gln Val Thr Thr Ala Lys Lys Leu
            500                 505                 510
Arg Gly Gly Val Val Phe Val Asp Glu Val Pro Lys Gly Leu Thr Gly
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Lys Leu Asp Ala Arg Lys Ile Arg Glu Ile Leu Ile Lys Ala Lys Lys
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<210> 4
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<212> PRT
<213> Artificial Sequence
<223> Description of Artificial Sequence: pGFP+luc* plasmid

<400> 4

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Phe Cys Leu Pro Val Phe Ala His Pro Glu Thr Leu Val Lys Val Lys
            20                  25                  30
Asp Ala Glu Asp Gln Leu Gly Ala Arg Val Gly Tyr Ile Glu Leu Asp
        35                  40                  45
Leu Asn Ser Gly Lys Ile Leu Glu Ser Phe Arg Pro Glu Glu Arg Phe
    50                  55                  60
Pro Met Met Ser Thr Phe Lys Val Leu Leu Cys Gly Ala Val Leu Ser
65                  70                  75                  80
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Arg Ile Asp Ala Gly Gln Glu Gln Leu Gly Arg Arg Ile His Tyr Ser
                85                  90                  95
Gln Asn Asp Leu Val Glu Tyr Ser Pro Val Thr Glu Lys His Leu Thr
            100                 105                 110
Asp Gly Met Thr Val Arg Glu Leu Cys Ser Ala Ala Ile Thr Met Ser
        115                 120                 125
Asp Asn Thr Ala Ala Asn Leu Leu Leu Thr Thr Ile Gly Gly Pro Lys
    130                 135                 140
Glu Leu Thr Ala Phe Leu His Asn Met Gly Asp His Val Thr Arg Leu
145                 150                 155                 160
Asp Arg Trp Glu Pro Glu Leu Asn Glu Ala Ile Pro Asn Asp Glu Arg
                165                 170                 175
Asp Thr Thr Met Pro Val Ala Met Ala Thr Thr Leu Arg Lys Leu Leu
            180                 185                 190
Thr Gly Glu Leu Leu Thr Leu Ala Ser Arg Gln Gln Leu Ile Asp Trp
        195                 200                 205
Met Glu Ala Asp Lys Val Ala Gly Pro Leu Leu Arg Ser Ala Leu Pro
    210                 215                 220
Ala Gly Trp Phe Ile Ala Asp Lys Ser Gly Ala Gly Glu Arg Gly Ser
225                 230                 235                 240
Arg Gly Ile Ile Ala Ala Leu Gly Pro Asp Gly Lys Pro Ser Arg Ile
                245                 250                 255
Val Val Ile Tyr Thr Thr Gly Ser Gln Ala Thr Met Asp Glu Arg Asn
            260                 265                 270
Arg Gln Ile Ala Glu Ile Gly Ala Ser Leu Ile Lys His Trp
        275                 280                 285
```

PATENT COOPERATION TREATY

PCT

REC'D 03 OCT 2001
PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

3

Applicant's or agent's file reference AP2969	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/FI00/00507	International filing date *(day/month/year)* 07.06.2000	Priority date *(day/month/year)* 07.06.1999
International Patent Classification (IPC) or national classification and IPC7 C12Q 1/02, C12Q 1/22, C12Q 1/00		
Applicant Lilius Esa-Matti et al		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of _____ sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
19.12.2000	20.09.2001
Name and mailing address of the IPEA/SE [illegible] Telefax 08-667 72 88	Authorized officer Carolina Palmcrantz/BS Telephone No. 08-782 25 00

Form PCT/IPEA/409 (cover sheet) (January 1998)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/FI00/00507

I. Basis of the report

1. With regard to the **elements** of the international application:*

☒ the international application as originally filed

☐ the description:
pages _____, as originally filed
pages _____, filed with the demand
pages _____, filed with the letter of _____

☐ the claims:
pages _____, as originally filed
pages _____, as amended (together with any statement) under article 19
pages _____, filed with the demand
pages _____, filed with the letter of _____

☐ the drawings:
pages _____, as originally filed
pages _____, filed with the demand
pages _____, filed with the letter of _____

☐ the sequence listing part of the description:
pages _____, as originally filed
pages _____, filed with the demand
pages _____, filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☐ The amendments have resulted in the cancellation of:

☐ the description, pages _____

☐ the claims, Nos. _____

☐ the drawings, sheet/fig _____

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2 (c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item I and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/FI00/00507

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-5	YES
	Claims		NO
Inventive step (IS)	Claims	1-5	YES
	Claims		NO
Industrial applicability (IA)	Claims	1-5	YES
	Claims		NO

2. Citations and explanations (Rule 70.7)

The present application pertains to a method to enable assessment of the growth rate and death rate of a microorganism within a chosen time period. The method involves introducing into said microorganism at least two reporter genes encoding luminescent, e.g. luciferase, and/or fluorescent products, e.g. green fluorescent protein (GFP). At least two of the following products are produced:

i) a stable product produced in a known proportion to the total amount of cells that are or have been alive,
ii) a product present in a known proportion to the amount of cells alive, and
iii) a stable product produced in a known proportion to the total amount of cells that have died.

The international search report revealed five documents of importance:

D1) File WPI, Derwent accession no. 1999-379000, ZH TETSUDO SOGO GIJUTSU KENKYUSHO: "Rapid identification of microorganism cell for measuring sterilization effect etc - involves using fluorescent pigments wich differ in their wavelengths for identifying dead and living cells"; & JP,A,11146798, 19990602, DW199932

D2) US 5164301 A (THOMAS E. THOMPSON ET AL), 17 November 1992 (17.11.92), column 4, lines 34-39, 42-56

.../...

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Box V

D3) Dialog Informaton services, File 5, BIOSIS, Dialog accession no. 11223430, BIOSIS no. 199800004762,Fratamico Pina M. et al: "Construction and characterization of Escherichia coli 0157:H7 strains expressing firefly luciferase and green fluorescent protein ant their use in survival studies"; & Journal of Food Protection 60 (10):p1167-1173 Oct.,1997

D4) WO 9814605 A1 (LOMA LINDA UNIVERSITY), 9 April 1998 (09.04.98), page 2, lines 4-7, 15-16

D5) US 5824468 A (SIEGFRIED SCHERER ET AL), 20 October 1998 (20.10.98), column 3, line 8-line 9, claim 5

D1 discloses the identification of dead and living cells using fluorescent pigments in order to measure e.g., the sterilization effect by pharmaceuticals for food.

D2 concerns a process for detecting microbial metabolism. The process involves a two dye fluorescence emission system which detects microbial growth (refer to column 4, lines 34-39).

D3 discloses the construction and characterization of Escherichia coli 0157:H7 strains expressing firefly luciferase and GFP. The strains may be used to monitor bacterial survival in foods and in a food processing environment.

D4 pertains to a luciferase and green fluorescent protein fusion gene. The fusion gene is useful as a double marker for monitoring gene expression in living cells and enzymatic activity (refer to page 2, lines 15-16).

From D1 and D2 it is considered to be known to measure microbial growth by using fluorescence dyes to differentiate between living and dead cells. The methods in D1 and D2 do not involve the use of two different reporter genes within one microorganism. Further, it is known from D3 and D4 to use two different markers, luciferase and GFP, within the same microorganism. However, neither D3 nor D4 discloses that GFP can be used as a measure of cells that have been or are alive, thus making it possible in combination with luciferase to discriminate between living and dead cells.

.../...

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Box V

Thus, it is not considered obvious to a person skilled in the art to use a luciferase-and GFP fusion gene as a double marker to differentiate between living and dead cells in order to measure the growth rate and death rate of a microorganism. Therefore, claims 1-5 are considered to be novel, to involve an inventive step and to have industrial applicability.

D5 relates to a detection procedure for bacteria of the genus Listeria. The procedure involves the use of a marker gene (refer to claim 5). D5 is considered to show the general state of the art and is not considered to be of particular relevance.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 00/00507

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12Q 1/02, C12Q 1/22, C12Q 1/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	File WPI, Derwent accession no. 1999-379000, ZH TETSUDO SOGO GIJUTSU KENKYUSHO: "Rapid identification of microorganism cell for measuring sterilization effect etc - involves using fluorescent pigments wich differ in their wavelengths for identifying dead and living cells"; & JP,A,11146798, 19990602, DW199932 --	1-5
Y	US 5164301 A (THOMAS E. THOMPSON ET AL), 17 November 1992 (17.11.92), column 4, lines 34-39, 42-56 --	1-5

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 Sept 2000	10.10.2000
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Palmcrantz/GH Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 00/00507

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Dialog Informaton services, File 5, BIOSIS, Dialog accession no. 11223430, BIOSIS no. 199800004762,Fratamico Pina M. et al: "Construction and characterization of Escherichia coli 0157:H7 strains expressing firefly luciferase and green fluorescent protein ant their use in survival studies"; & Journal of Food Protection 60 (10):p1167-1173 Oct.,1997 --	1-5
Y	WO 9814605 A1 (LOMA LINDA UNIVERSITY), 9 April 1998 (09.04.98), page 2, lines 4-7, 15-16 --	1-5
A	US 5824468 A (SIEGFRIED SCHERER ET AL), 20 October 1998 (20.10.98), column 3, line 8 - line 9, claim 5 --	1-5

INTERNATIONAL SEARCH REPORT

Information on patent family members

01/08/00

International application No.
PCT/FI 00/00507

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5164301 A	17/11/92	EP 0558827 A,B	08/09/93
		JP 5137594 A	01/06/93
WO 9814605 A1	09/04/98	AU 4500497 A	24/04/98
		EP 0934425 A	11/08/99
		US 5976796 A	02/11/99
US 5824468 A	20/10/98	DE 19517940 A	21/11/96
		EP 0743366 A	20/11/96